The NAMPT-mediated NAD salvage pathway in cancer cell metabolism and its regulation by resveratrol

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Dissertation

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Nicotinamide adenine dinucleotide (NAD) is a key regulator of several metabolic and signaling pathways that are relevant in cancer cell survival. Cancer cells have an increased energy demand associated with an increased NAD turnover. Nicotinamide phosphoribosyltransferase (NAMPT), a key enzyme of the NAD salvage pathway, plays a crucial role in maintaining the intracellular NAD levels and in regulating the activity of NAD-dependent enzymes, such as sirtuins (SIRTs). The inhibition of NAMPT activity and the use of phytochemicals, such as resveratrol, represent novel therapeutic approaches in cancer therapy. Based on these facts, this thesis aimed to investigate **(1)** the chemotherapeutic potential and molecular mechanisms of FK866, a specific NAMPT inhibitor, and resveratrol on hepatocarcinoma cells and to find out whether there are differences compared to primary human hepatocytes; **(2)** to address the impact of NAMPT inhibition on the energy metabolism in cancer cells; and **(3)** to investigate the roles of NAMPT and SIRT1 in resveratrol´s mode of action and chemotherapeutic effects. This work demonstrates that FK866 and resveratrol possess potent chemotherapeutic effects in hepatocarcinoma cells which were absent in human hepatocytes. Hepatocarcinoma cells display a dysregulation in the AMP-activated kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling as well as in the NAMPT-mediated NAD salvage pathway compared to human hepatocytes. FK866-induced NAMPT inhibition induces ATP depletion associated with AMPK activation and mTOR inhibition whereas resveratrol induces caspase3-mediated apoptosis that is not dependent on NAMPT and SIRT1 function. NAMPT and SIRT1 are differentially regulated by resveratrol in hepatocarcinoma cells and human hepatocytes. This work also reveals that resveratrol activates p53-induced cell cycle arrest in hepatocarcinoma cells which is partly mediated by SIRT1 inhibition. In summary, this thesis provides new insight into the role of the NAMPT-mediated NAD salvage pathway in energy metabolism and characterized FK866 and resveratrol as promising potential chemotherapeutic agents for treatment of hepatocellular carcinoma.

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ABBREVIATIONS

4E-BP1	eukaryotic initiation factor 4E binding protein 1
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
An	Annexin
ATP	adenosine triphosphate
BAT	brown adipose tissue
BCL6	B-cell lymphoma 6 protein
BESTO	β cell-specific Sirt1-overexpressing
BMI	body mass index
BP	blood pressure
BSA	bovine serum albumin
CAD	coronary artery disease
cADPR	cyclic ADP-ribose
cDNA	complementary desoxyribonucleic acid
CI	confidence interval
CKD	chronic kidney disease
COX	cyclooxygenases
Cpm	counts per minute
CRP	C-reactive protein
CT	computed tomography
CVD	coronary vascular disease
DBP	diastolic blood pressure
DENA	diethylnitrosamine
DNA	desoxyribonucleic acid
EDTA	ethylendiamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
eNampt	extracellular form of Nampt
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAD	flavin adenine dinucleotide
$FADH_2$	flavin adenine dinucleotide dihydrate

FITC	fluorescein isothiocyanate
FoxO	forkhead box 'Other'
FPG	fasting plasma glucose
FRD	fructose-rich diet
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDM	gestational diabetes mellitus
GH	growth hormone
GLUT	glucose transporter
GSIS	glucose-stimulated insulin secretion
HbA1C	glycated hemoglobin
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC1	hypermethylated in cancer 1
HIF-1α	hypoxia inducible factor 1 alpha
HIF-2α	hypoxia-inducible factor 2 alpha
HOMA-IR	homeostatic model assessment – insulin resistance
HPLC	high-pressure liquid chromatography
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IDO	indoleamine-2,3,-dioxigenase
Ig	immunoglobulin
IGF	insulin-like growth factor
IGT	impaired glucose tolerance
IL	interleukin
iNAMPT	intracellular form of NAMPT
IR	insulin receptor
K	lysine
LDHA	lactate dehydrogenase A
LKB1	liver kinase B1
MAPK	mitogen-activated protein kinase
MART	mono-ADP ribosyltransferase
MCP	monocyte chemoattractant protein
miRNA	micro RNA
MMP	matrix-metalloproteinases

MNNG	1-methyl-3-nitro-1-nitrosoguanidinium
mRNA	messenger ribonucleid acid
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NA	nicotinic acid
NAAD	nicotinic acid adenine dinucleotide
NAADP	nicotinate adenine dinucleotide phosphate
NAAM	nicotinic acid mononucleotide
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrate
NADS	NAD synthase
NAFLD	non-alcoholic fatty liver disease
NAM	nicotinamide
NAMPT	nicotinamide phosphoribosyltransferase
NAPRT	nicotinic acid phosphoribosyltransferase
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor κB
NGT	normal glucose tolerance
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide/nicotinic acid mononucleotide adenylyltransferase
NR	nicotinamide riboside
NRK	nicotinamide riboside kinase
NSPC	neuronal stem neural stem/progenitor cells
OGTT	oral glucose tolerance test
OR	odds ratio
p70S6K	p70 ribosomal S6 kinase
PAI	plasminogen activator inhibitor
PARP	poly (ADP-ribose) polymerase
PBEF	pre B-cell colony enhancing factor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCOS	polycystic ovary syndrome
PGC1-α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PHH	primary human hepatocytes

PI	propidium iodide
PI3K	phosphoinositide-3-kinase
PKB/AKT	protein kinase B
PKM1/2	pyruvate kinase 1 and 2
PPAR	peroxisome proliferator-activated receptor
PP_i	pyrophosphate
PPP	pentose phosphate pathway
PRPP	5-phosphoribosyl-1-pyrophosphate
Rib-5-P	ribose-5-phosphate
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SAT	subcutaneous adipose tissue
SBP	systolic blood pressure
SCF	stem cell factor
SEM	standard error of the mean
Ser	serine
SFM	serum free medium
Sir2	silent information regulator 2
SIRTs	sirtuins
SMC	smooth muscle cell
SNPs	single nucleotide polymorphisms
SREBP	sterol regulatory element-binding protein
STAT3	signal transducer and activator of transcription 3
T2D	type 2 diabetes mellitus
TC	total cholesterol
TCA	tricarboxylic acid cycle
TDO	tryptophan-2,3,-dioxigenase
TG	triglycerides
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAF6	TNF receptor associated factor
TSA	Trichostatin A
TSC2	tuberous sclerosis complex-2
VAT	visceral adipose tissue
VCAM	vascular cell adhesion molecule

VEGF	vascular endothelial growth factor
WAT	white adipose tissue
WHR	waist-to-hip ratio

SUMMARY

For almost a century it has been known that cancer cells show fundamental differences in their central metabolic and signaling pathways compared to non-transformed cells. Already in the 1920s, Otto Warburg found that under aerobic conditions, tumor tissue metabolizes approximately tenfold more glucose to lactate in a given time than normal tissue. The so-called Warburg effect describes that cancer cells under normoxic conditions have a high glycolytic rate accompanied by reduced oxidative phosphorylation. These findings have influenced the scientific community significantly. Today it is known that due to changes in central metabolic pathways and rapid cell proliferation cancer cells have an increased energy demand compared to normal cells. These metabolic alterations require the redox co-factor nicotinamide adenine dinucleotide (NAD). Thus, cancer cells have an increased NAD turnover due to their need for continuous energy supply. Throughout this thesis, the term NAD will be used instead of NAD^+ (the oxidized form).

Besides its role in cellular redox reactions, NAD plays a pivotal role as substrate for NAD-dependent enzymes, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs), ADP-ribosyl cyclases/CD38 and sirtuins. These enzymes regulate different biological processes, including transcription, cell cycle progression, apoptosis, intracellular calcium mobilization and metabolic pathways – processes that undergo fundamental changes during malignant transformation. Thus, NAD has emerged as one of the most important links between regulatory and bioenergetic processes. The characteristic features of cancer cell metabolism and the importance of the NAD salvage pathway in cancer cell biology are described in **Chapter 1**. NAD-dependent enzymes continuously consume NAD and thereby degrade NAD with the concomitant release of nicotinamide, which has a negative feedback function. Thus, constant NAD recycling is an absolute requirement for cell survival and cell growth, especially for rapidly growing cells. The permanent conversion of nicotinamide to NAD is mediated by nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the mammalian NAD biosynthesis starting from nicotinamide. NAMPT catalyzes that rate limiting step in the NAD salvage pathway generating nicotinamide mononucleotide (NMN) which is then converted into NAD by isoenzymes of nicotinamide mononucleotide adenylyltransferases (NMNATs) 1-3. NAMPT can be found intracellularly (iNAMPT) and extracellularly in supernatant and plasma (eNAMPT). Both, intracellular and extracellular NAMPT seem to be responsible for its relevance in a variety of human diseases, including cancer progression. To maintain intracellular NAD supply tumor cells often overexpress NAMPT.

In this context, clinical studies reported increased serum NAMPT levels in cancer patients with a positive correlation between either tissues or circulating levels and stage progression.

NAMPT regulates the activity of NAD-dependent enzymes, such as the deacetylase sirtuin 1 (SIRT1). SIRT1 belongs to the class III of histone deacetylases (also called lysine deacetylases) and besides histone proteins, SIRT1 also regulates the expression and activity of non-histone proteins and transcription factors including p53, forkhead box 'Other' (FoxO) proteins, hypoxia-inducible factor 2α (HIF-2α), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), Ku70 and others. Thus, SIRT1 is implicated in a number of metabolic pathways and biological processes that are important in cancer cell metabolism. Several studies postulated a tumor promotor function for SIRT1. Additionally, different cancer types including hepatocellular carcinoma (HCC) show an increased SIRT1 expression and activity. However, the expression and regulation of NAMPT in HCC has not been described so far. HCC is the second leading cause of cancer-related deaths worldwide and treatment options are limited. To understand the mechanisms of tumor progression is essential for the development of novel therapy approaches. Therefore, targeting NAMPT activity and reducing NAD salvage represent a promising chemotherapeutic option in HCC cancer therapy.

The uncontrolled proliferation presents a significant bioenergetic challenge to cancer cells. As key physiological energy sensor, AMP-activated protein kinase (AMPK) is a major regulator of cellular energy homeostasis that translates changes in glucose availability and fluctuation of energy to mammalian target of rapamycin (mTOR). AMPK activation has been shown to inhibit cell proliferation and to abrogate growth of HCC xenografts. As a conserved serine/threonine protein kinase, mTOR occupies a central role in the regulation of proliferation, differentiation, cell growth and angiogenesis. mTOR down-stream targets are the ribosomal protein S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), both responsible for protein synthesis and cell growth. mTOR suppresses apoptosis and is frequently hyperactivated in human cancers. Therefore, a dysregulation of the AMPK/mTOR signaling pathway has been assumed to play an essential role in cancer development. Thus, altered metabolism has now been recognized as one of the hallmarks of cancer and has thus become an attractive target for cancer therapy. Reducing NAMPT activity and intracellular NAD content may represent a novel therapeutic concept to influence cellular energy metabolism and to regulate the AMPK/mTOR signaling pathway.

Furthermore, emerging evidence indicated the use of natural agents as cancer therapy. Phytochemicals, such as resveratrol, have received increasing attention as they exert chemopreventive and chemotherapeutic potential. In different screening assays as well as in animal studies, resveratrol has been shown to activate AMPK and SIRT1. However, further studies argued whether resveratrol activates SIRT1 directly or not. Additionally, the role of NAMPT in the chemotherapeutic action of resveratrol in cancer cells was not yet known at the beginning of this thesis.

Based on these facts, the hypothesis was proposed that targeting NAMPT activity by pharmacological inhibition reduces cell viability of hepatocarcinoma cells. Therefore, the aims of my thesis were **(1)** to investigate the chemotherapeutic potential and molecular mechanisms of the polyphenol resveratrol and of FK866, a specific NAMPT inhibitor, on hepatocarcinoma cells and to find out whether there are differences compared to non-cancerous primary human hepatocytes; **(2)** to address the impact of a pharmacological NAMPT inhibition on the energy metabolism and the AMPK/mTOR pathway in hepatocarcinoma cells and human hepatocytes; and **(3)** to investigate the functional roles of NAMPT and SIRT1 in resveratrol´s mode of action and chemotherapeutic effects and to compare them between both cell models (hepatocarcinoma cells and primary human hepatocytes). The results obtained in my thesis provide new insight into the role of the NAMPT-mediated NAD salvage pathway in cancer cell metabolism and the underlying molecular mechanisms of FK866 and resveratrol in hepatocarcinoma cells.

Chapter 2 gives an overview of the physiological and pathophysiological functions of NAMPT and its relevance in different diseases.

The experimental study presented in **Chapter 3** aimed at investigating the effects of a pharmacological NAMPT inhibition by FK866 on cancer cell viability and asked whether NAD depletion induces energy stress that may activate AMPK and downregulate mTOR signaling. It also targeted the question whether non-cancerous human hepatocytes are less sensitive to a NAMPT inhibition than cancer cells. To determine whether the observed FK866 effects are dependent on NAMPT enzymatic function, the cells were co-stimulated with the NAMPT enzyme product, NMN, to reverse cellular NAD depletion. First, we aimed to identify differences in the energy metabolism in hepatocarcinoma cells and human hepatocytes and found that AMPK was significantly less activated in hepatocarcinoma cells compared to non-cancerous human hepatocytes. In contrast components of the mTOR complex1 (mTORC1) cascade, such as mTORC1, p70S6K and 4E-BP1, were higher expressed in hepatocarcinoma cells than in human hepatocytes. This supports the

hypothesis that a dysregulation of the AMPK/mTOR pathway may contribute to the development of HCC.

In further analysis, the effects of a FK866-mediated NAMPT inhibition on energy metabolism and cell viability of hepatocarcinoma cells and human hepatocytes were studied. We could show that FK866 rapidly reduced NAD levels and led to delayed cell death after 72h associated with ATP depletion in hepatocarcinoma cells. Furthermore, FK866-induced NAD reduction led to a decreased activity of NAD-dependent lysine deacetylases as measured by an increased global acetylation of protein lysine residues by immunoblot. In subsequent analysis, FK866-treated hepatocarcinoma cells displayed increased AMPK activation and inhibition of mTOR signaling. The co-administration of NMN abrogated the effects of FK866 on cell viability and on the AMPK/mTOR signaling pathway suggesting that these effects were mediated by reduced NAMPT enzymatic function. Interestingly, the same concentration of FK866 used in hepatocarcinoma cells did not affect NAD production and the AMPK/mTOR signaling cascade in non-cancerous human hepatocytes indicating a reduced sensitivity of non-cancerous cells to FK866. In summary, FK866 induced delayed cell death in hepatocarcinoma cells by reducing the cellular ATP level, activating AMPK and downregulating mTOR signaling. The results of this work also suggest that FK866 has low cytotoxic effects on primary hepatocytes.

The polyphenol resveratrol has been described to possess chemotherapeutic properties in different cancer types including HCC. In **Chapter 4**, we investigated the molecular mechanisms of resveratrol and attempted to identify the relevance of NAMPT and SIRT1 protein function in resveratrol´s mode of action in hepatocarcinoma cells and in human hepatocytes. We asked whether NAMPT and SIRT1 are involved in resveratrol´s chemotherapeutic function. Resveratrol decreased cell viability and selectively induced p53 phosphorylation in hepatocarcinoma cells but not in human hepatocytes. Further, moderate doses of resveratrol led to cell cycle arrest in the S- and G2/M-phase of hepatocarcinoma cells in a p53-dependent manner. In addition, p53-deficient hepatocarcinoma cells did not show changes in cell cycle distribution but were more sensitive to apoptosis induction by resveratrol than p53 wild-type hepatocarcinoma cells. Resveratrol induced caspase-3-mediated apoptosis in all hepatocarcinoma cell lines indicating that resveratrol-induced apoptotic effects did not depend on p53 function. Measuring the basal expression of NAMPT and SIRT1, we found that both proteins were oppositely expressed in hepatocarcinoma cells and human hepatocytes. In particular, hepatocarcinoma cells expressed lower levels of NAMPT mRNA and protein compared to human hepatocytes. However, despite lower NAMPT protein levels, hepatocarcinoma cells

possessed higher basal NAMPT activity than non-cancerous hepatocytes. In contrast, the NAD-dependent deacetylase SIRT1 was significantly higher expressed in hepatocarcinoma cells compared to human hepatocytes.

In subsequent studies, we investigated the functional roles of NAMPT and SIRT1 in resveratrol´s mode of action in human hepatocytes and hepatocarcinoma cells. The present study could demonstrate that resveratrol positively regulated NAMPT activity and NAD production in non-cancerous human hepatocytes without affecting NAMPT protein expression, thus indicating beneficial substrate conditions for NAD-dependent enzymes. It may be assumed that post-translational modifications of the NAMPT protein play a role in NAMPT´s enzymatic regulation and that resveratrol potentially influences such reactions.

Interestingly, in contrast to our findings in human hepatocytes, NAMPT activity and SIRT1 protein expression were negatively regulated by resveratrol in hepatocarcinoma cells. High doses of resveratrol reduced NAMPT enzymatic activity and increased NAMPT release. The release of NAMPT into the supernatant was time-dependent and associated with increased *NAMPT* mRNA expression levels, suggesting a compensatory mechanism to maintain stable NAMPT protein levels. It is conceivable, that the resveratrol-mediated *NAMPT* mRNA induction and NAMPT release are induced as a stress-response reaction. Recent studies support this hypothesis as they found that different stress signals induced *NAMPT* gene expression and increased NAMPT serum levels.

Furthermore, resveratrol-treated hepatocarcinoma cells displayed reduced SIRT1 protein levels. SIRT1 targets and deacetylates the tumorsuppressor protein p53, thus leading to its inhibition and promoting tumorigenesis. We could demonstrate, that hepatocarcinoma cells treated with resveratrol showed increased p53 acetylation at lysine residue 382 (K382), a main target site of SIRT1, indicating a potentially reduced SIRT1 activity in these cells. p53 hyperacetylation leads to its transcriptional activation and induction of the downstream target p21/WAF1/Cip1 which was significantly increased in hepatocarcinoma cells in our study. To investigate whether the effects of resveratrol were mediated by the observed reduced NAMPT activity and NAD synthesis, cells were co-treated with the NAMPT enzymatic product, NMN. Interestingly, NMN did not ameliorate resveratrol-induced cell cycle arrest and apoptosis suggesting that the availability of NMN or NAD were not limiting factors in this scenario. To find out whether the reduced SIRT1 protein function was involved in the cellular effects of resveratrol in hepatocarcinoma cells, experiments on SIRT1 overexpression were performed. Our results revealed that a SIRT1 overexpression significantly decreased the resveratrol-induced cell

cycle arrest by reducing p53 acetylation (K382) but failed to abrogate resveratrol-induced apoptosis. This suggests an important role for SIRT1 in p53-induced cell cycle regulation but not in apoptotic mechanisms. Furthermore, increased SIRT1 expression also reversed resveratrol-induced NAMPT release. This indicates a regulatory function for SIRT1 in the mechanism of NAMPT secretion whereas the underlying mechanisms are not known.

To conclude this study, resveratrol positively regulated NAMPT activity and NAD levels in non-cancerous human hepatocytes but negatively influenced NAMPT and SIRT1 protein function in hepatocarcinoma cells. Further, SIRT1 was shown to be involved in p53-mediated cell cycle arrest and NAMPT secretion. However, neither NAMPT nor SIRT1 were involved in the resveratrol-mediated apoptotic effects on hepatocarcinoma cells. Thus, several collective activities, rather than just a single effect, may account for the anticancer properties of resveratrol.

In summary, this work demonstrates that a FK866-induced NAMPT inhibition reduces the cell viability of hepatocarcinoma cells and shows the importance of the NAMPT-mediated NAD salvage pathway for the energy metabolism in cancer cells. These results verify that hepatocarcinoma cells due to a higher basal NAMPT activity are more sensitive to a FK866-mediated NAMPT inhibition than non-cancerous human hepatocytes. Resveratrol was shown to reduce NAMPT and SIRT1 activity associated with an increased cell cycle arrest selectively in hepatocarcinoma cells as compared to non-cancerous human hepatocytes.

This thesis contributes to a better understanding of the molecular mechanisms and potential therapeutic functions of FK866 and resveratrol in the pathogenesis of hepatocellular carcinoma.

ZUSAMMENFASSUNG

Die unkontrollierte Teilung von Krebszellen stellt eine große Herausforderung an deren Energiestoffwechsel dar. In Folge dessen kommt es in Krebszellen zu gravierenden Veränderungen in verschiedenen Signal-und Stoffwechselwegen. Bereits 1924 konnte Otto Warburg zeigen, dass Tumorgewebe trotz aerober Bedingungen 10-mal so viel Glukose zu Laktat umsetzt als gesundes Gewebe. Warburg nannte diesen Effekt aerobe Glykolyse. Das bedeutet, dass Krebszellen auch in Anwesenheit von genügend Sauerstoff einen ausgeprägten glykolytischen Stoffwechsel besitzen und eine verminderte oxidative Phosphorylierung. Der veränderte Stoffwechsel sowie die gesteigerte Zellteilung und Proliferation führt in Krebszellen zu einem deutlich höheren Energiebedarf. Um diesen außerordentlichen Energiebedarf zu decken, sind Krebszellen auf eine kontinuierliche Verfügbarkeit des Redox-Kofaktors Nikotinamidadenindinukleotid (NAD) angewiesen und besitzen daher einen deutlich höheren NAD Umsatz als normale Zellen. In dieser Arbeit wird die Bezeichnung NAD anstelle von NAD^+ verwendet.

Neben seiner Tätigkeit als Koenzym bei Redox-Reaktionen besitzt NAD ebenfalls eine wichtige Funktion als Substrat von NAD-abhängigen Enzymen, wie z.B Poly-(ADP-ribose)-Polymerasen (PARPs), Mono-ADP-Ribosyltransferasen (MARTs), ADP-Ribosylcyclasen/CD38 und Sirtuinen (SIRTs). NAD-abhängige Enzyme sind an der Regulation verschiedener Stoffwechselprozesse beteiligt und spielen eine wichtige Rolle unter anderem bei der Transkription, dem Zellzyklus und der Apoptose – Prozesse, die besonders während der Tumorgenese wesentlichen Veränderungen unterliegen. Die charakteristischen Veränderungen im Stoffwechsel von Krebszellen sowie die Bedeutung von NAD werden in **Kapitel 1** dieser Arbeit erläutert.

NAD wird kontinuierlich von NAD-abhängigen Enzymen verbraucht, indem sie NAD abbauen und gleichzeitig Nikotinamid freisetzen. Daher ist die ständige Wiedergewinnung von NAD aus Nikotinamid besonders für Krebszellen von Bedeutung, da es das Zellüberleben und Krebswachstum sichert. Die permanente Umwandlung von Nikotinamid zu NAD wird durch die Nikotinamidphosphoribosyltransferase (NAMPT), einem Schlüsselenzym der NAD-Biosynthese, vermittelt. NAMPT synthetisiert Nikotinamid-Mononukleotid (NMN), welches danach durch die Isoenzyme Nikotinamid-Mononukleotid-Adenylyltransferase (NMNAT) 1-3 weiter zu NAD umgewandelt wird. NAMPT konnte sowohl intrazellulär (iNAMPT) als auch extrazellulär bzw. im Plasma (eNAMPT) nachgewiesen werden. Jedoch sind die strukturellen Unterschiede beider Formen bis heute nicht bekannt. Man geht aber davon aus, dass die unterschiedlichen

Funktionen von eNAMPT und iNAMPT bei der Entstehung verschiedener Krankheiten, auch bei der Tumorprogression, eine Rolle spielen. Um eine kontinuierliche NAD-Versorgung zu gewährleisten, wird NAMPT in Krebszellen häufig überexprimiert. Klinische Studien zeigten, dass der NAMPT-Serumspiegel in Krebspatienten nicht nur erhöht war, sondern auch mit dem Grad der Tumorerkrankung korrelierte.

Als NAD-produzierendes Enzym reguliert NAMPT die Aktivität NAD-abhängiger Enzyme, beispielsweise der Deacetylase Sirtuin 1 (SIRT1). Obwohl SIRT1 zu der Klasse III der Histon-Deacetylasen (auch Lysin-Deacetylasen genannt) gehört, reguliert es neben Histon-Proteinen auch die Expression und Aktivität von Nicht-Histon-Proteinen und Transkriptionsfaktoren, wie z.B. p53, Proteine der *forkhead box 'Other'* (FoxO) Familie, *hypoxia-inducible factor* 2α (HIF-2α), *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (PGC1-α) und Ku70. Damit beeinflusst SIRT1 eine Vielzahl von Stoffwechselwegen und biologischen Prozessen, die vor allem während der Tumorentstehung von Bedeutung sind. In verschiedenen Tumorgeweben, unter anderem auch im hepatozellulären Karzinom (HCC), konnte eine verstärkte SIRT1-Expression nachgewiesen werden. Die Expression und Regulation von NAMPT im HCC-Gewebe ist jedoch nicht bekannt. Das HCC gehört zu der weltweit zweithäufigsten Krebs-Todesursache und dessen Behandlungsmöglichkeiten sind derzeit sehr begrenzt. Daher ist das Verständnis der molekularen Mechanismen der Tumorgenese essentiell für die Entwicklung neuer Therapieansätze. Die Inhibierung der NAMPT-Enzymaktivität stellt eine vielversprechende neue Therapieoption bei der Behandlung des HCCs dar.

Der zelluläre Energiehaushalt wird maßgeblich durch die AMP-aktivierte Proteinkinase (AMPK) reguliert. Als physiologischer Sensor übermittelt die AMPK Veränderungen in der Glukoseverfügbarkeit und Schwankungen im Energiehaushalt an das *mammalian target of rapamycin* (mTOR). Die Aktivierung der AMPK zeigte in mehreren Studien eine anti-proliferative Wirkung und verminderte das Krebswachstum in HCC-Xenograft-Tieren. Als hochkonservierte Serin/Threonin-Proteinkinase spielt mTOR eine wichtige Rolle in der Regulation von Proliferation, Differenzierung und Vermehrung der Zelle. mTOR kontrolliert dabei die Translation von Proteinen und Transkriptionsfaktoren und sichert somit das Zellüberleben. Zu seinen Zielmolekülen gehört unter anderem die ribosomale S6 Proteinkinase (p70S6K) und das eukaryotische Translationsinitiationsfaktor 4E (eIF4E) Bindeprotein1 (4E-BP1). Über diese Signalkaskade kommt es zur Stimulation der Proliferation, des Zellwachstums und der Angiogenese sowie zur Unterdrückung der Apoptose. In Folge dessen ist mTOR in malignen Tumoren häufig überaktiviert und gilt daher als interessantes und vielversprechendes Zielprotein in der Krebstherapie. Man

vermutet, dass eine Dysregulation des AMPK/mTOR-Signalwegs eine essentielle Rolle bei der Krebsentstehung und bei der Tumorprogression spielt. Die pharmakologische Inhibierung der NAMPT-Enzymaktivität und die damit verbundene Reduzierung der intrazellulären NAD-Spiegel stellen einen neuen Ansatzpunkt in der Krebstherapie dar, um den Energiehaushalt der Krebszelle zu beeinflussen und den AMPK/mTOR-Signalweg zu regulieren.

Darüber hinaus erregten in den letzten Jahren sekundäre Pflanzenstoffe, wie z.B. Resveratrol, in der Krebsforschung großes wissenschaftliches Interesse. So überzeugte Resveratrol in verschiedenen Versuchsmodellen sowohl mit seiner chemopräventiven als auch mit seiner chemotherapeutischen Wirksamkeit. In verschiedenen *in vitro* Screeningassays sowie in verschiedenen Tiermodellen wurde Resveratrol als natürlicher Aktivator von AMPK und SIRT1 identifiziert. Zahlreiche Studien folgten, die die SIRT1-aktivierende Wirkung von Resveratrol zum Thema hatten, jedoch aber widersprüchliche Ergebnisse hervorbrachten. Daher ist umstritten, ob Resveratrol SIRT1 direkt reguliert oder indirekt durch z.B. eine Erhöhung des zellulären NAD-Spiegels. Zudem war zu Beginn dieser Arbeit nicht bekannt, welche Rolle NAMPT in der chemotherapeutischen Wirkung von Resveratrol auf Hepatokarzinomzellen spielt.

Basierend darauf, stellte ich die Hypothese auf, dass eine Inhibierung der NAMPT-Enzymaktivität die Viabilität von Hepatokarzinomzellen vermindert. Um diese Hypothese zu prüfen, sollten in der vorliegenden Arbeit (**1**) die chemotherapeutische Wirkung von Resveratrol sowie die molekularen Mechanismen von FK866, einem NAMPT-spezifischen Inhibitor, in Hepatokarzinomzellen und nicht-kanzerogenen primären humanen Hepatozyten untersucht werden. Zudem sollte **(2)** die Frage beantwortet werden, ob eine NAMPT-Inhibition den Energiehaushalt und den AMPK/mTOR-Signalweg beeinflusst und ob Hepatokarzinomzellen sensitiver auf eine NAMPT-Inhibierung reagieren als normale Hepatozyten. In weiteren Analysen sollte **(3)** die Funktion von NAMPT und SIRT1 in der Wirkungsweise von Resveratrol untersucht und in beiden Zellmodellen (Hepatokarzinomzellen und humane Hepatozyten) verglichen werden. Die Ergebnisse dieser Arbeit tragen zu einem besseren Verständnis der pharmakologischen NAMPT-Inhibition im Krebsstoffwechsel bei und geben Aufschluss über die molekularen Mechanismen der chemotherapeutischen Wirkung von FK866 und Resveratrol in Hepatokarzinomzellen.

Ein Überblick über die physiologischen und pathophysiologischen Funktionen von NAMPT sowie seine Relevanz bei diversen Erkrankungen wird in **Kapitel 2** dieser Arbeit gegeben.

Kapitel 3 dieser Arbeit beschäftigt sich mit den Auswirkungen einer FK866-vermittelten NAMPT-Inhibierung auf den Energiehaushalt und das Zellwachstum von Hepatokarzinomzellen. Dafür untersuchten wir zunächst basale Unterschiede im Energiestoffwechsel von Hepatokarzinomzellen und humanen Hepatozyten. In diesem Zusammenhang zeigten Hepatokarzinomzellen eine verminderte AMPK-Aktivierung sowie eine deutlich höhere Aktivität des mTOR-Komplex-1-Signalwegs. Diese Ergebnisse unterstützen die Annahme, dass eine Dysregulation des AMPK/mTOR-Signalwegs bei der Entstehung des HCC eine wichtige Rolle spielt.

In weiteren *in-vitro* Analysen untersuchten wir die Effekte der pharmakologischen NAMPT-Inhibierung auf die Viabilität und den Energiestoffwechsel von Hepatokarzinomzellen und verglichen sie mit denen in humanen Hepatozyten. Die Behandlung der Hepatokarzinomzellen mit FK866 führte zu einer raschen Senkung des zellulären NAD-Spiegels sowie zu einem verzögerten Eintritt des Zelltods nach 72h, der mit einer Reduktion des zellulären ATP-Spiegels korrelierte. Zugleich zeigten die FK866-behandelten Zellen eine verminderte Aktivität von NAD-abhängigen Deacetylasen, was zu einem Anstieg der Acetylierung von Lysinresten an Proteinen führte. In weiteren Analysen führte die FK866-induzierte NAD-Abnahme zur Aktivierung der AMPK und zur Hemmung des mTOR-Signalwegs in Hepatokarzinomzellen. Um festzustellen, ob die FK866-vermittelten Effekte von der NAMPT-Enzymaktivität abhängig sind, wurden die Zellen mit FK866 und dem NAMPT-Enzymprodukt NMN ko-stimuliert. Die Ergebnisse zeigten, dass die Zugabe von NMN nicht nur die Effekte auf den AMPK/mTOR-Signalweg, sondern auch auf den FK866-induzierten Zelltod, aufheben konnten. Damit konnte nachgewiesen werden, dass die zellulären Effekte von FK866 durch eine verminderte NAMPT-Enzymaktivität vermittelt werden. Interessanterweise zeigten humane Hepatozyten, die unter den gleichen Bedingungen mit FK866 behandelt wurden, keine signifikanten Veränderungen in ihrem NAD-Spiegel oder im AMPK/mTOR-Signalweg. Dies macht deutlich, dass primäre Hepatozyten eine geringere Sensitivität gegenüber FK866 aufweisen. Zusammenfassend konnten diese Ergebnisse belegen, dass eine FK866-induzierte NAMPT-Inhibierung zu einer Reduzierung der Viabilität von Hepatokarzinomzellen führte. Dies war mit einem Abfall des zellulären ATP-Spiegels und der Aktivierung der AMPK und Hemmung des mTOR-Signalwegs assoziiert. Des

Weiteren bestätigten diese Ergebnisse, dass Hepatokarzinomzellen sensitiver auf eine NAMPT-Inhibierung reagieren als primäre Hepatozyten.

Der sekundäre Pflanzenstoff Resveratrol konnte bereits in unterschiedlichen *in vitro* und *in vivo* Studien sowohl seine chemopräventive als auch chemotherapeutische Wirkung zeigen. **Kapitel 4** dieser Arbeit gibt Aufschluss über die molekulare Wirkungsweise von Resveratrol in Hepatokarzinomzellen und primären Hepatozyten und geht der Frage nach welche Rolle NAMPT und SIRT1 bei der chemotherapeutischen Wirkung von Resveratrol spielen.

Die Ergebnisse dieser Arbeit zeigten, dass Resveratrol die Viabilität von Hepatokarzinomzellen signifikant reduzierte und zu einer verstärkten Phosphorylierung von p53 führte. Moderate Dosen dieses Polyphenols induzierten einen p53-abhängigen Zellzyklusarrest in der S- und G2/M-Phase in Hepatokarzinomzellen. In p53-defizienten Hepatokarzinomzellen konnte hingegen keine Veränderung im Zellzyklus festgestellt werden. Jedoch induzierte Resveratrol in diesen Zellen stärker Apoptose als in p53-Wildtyp Hepatokarzinomzellen. Zudem führte Resveratrol in allen Hepatokarzinomzelllinien zu einer Caspase-3-vermittelten Apoptose, was darauf hindeutet, dass die Resveratrol-induzierte apoptotische Wirkung p53-unabhängig vermittelt wird. Beim Vergleich der Expression von NAMPT und SIRT1 in Hepatokarzinomzellen und humanen Hepatozyten zeigte sich, dass beide Proteine gegensätzlich exprimiert wurden. So wiesen Hepatokarzinomzellen eine signifikant verminderte *NAMPT*-mRNA- und NAMPT-Protein-Expression gegenüber humanen Hepatozyten auf. Interessanterweise besaßen jedoch die Hepatokarzinomzellen, trotz niedrigerer NAMPT-Proteinspiegel, eine höhere basale NAMPT-Enzymaktivität als humane Hepatozyten. Die Expression der Deacetylase SIRT1 zeigte hingegen eine signifikant stärkere Expression in Hepatokarzinomzellen als in primären Hepatozyten.

In weiteren Experimenten sollte untersucht werden, welche Funktion NAMPT und SIRT1 in der Wirkungsweise von Resveratrol in Hepatokarzinomzellen und humanen Hepatozyten haben und ob sie eine Rolle bei dessen chemotherapeutischer Wirkung spielen. Wir konnten zeigen, dass Resveratrol in primären Hepatozyten einen positiven Effekt auf die NAMPT-Aktivität und die damit verbundenen zellulären NAD-Spiegel ausübte, ohne die intrazellulären NAMPT-Proteinspiegel zu beeinflussen. Es ist daher anzunehmen, dass Resveratrol eventuell zu post-translationalen Veränderungen am NAMPT-Protein führt, die die Enzymaktivität beeinflussen. In Hepatokarzinomzellen hingegen führte die Resveratrol-Behandlung zu einer verminderten NAMPT-

Enzymaktivität sowie zu einer verstärkten Freisetzung von NAMPT in den Zellüberstand. Die vermehrte NAMPT-Freisetzung war mit einer erhöhten *NAMPT*-mRNA-Expression assoziiert, was auf eine kompensatorische Funktion zur Aufrechterhaltung der intrazellulären NAMPT-Proteinspiegel hindeutet. Es ist daher anzunehmen, dass NAMPT verstärkt als Stressantwort von den Zellen exprimiert und freigesetzt wird. Aktuelle Studien unterstützen diese Annahme. Sie konnten zeigen dass verschiedene Arten von Zellstress die *NAMPT*-Genexpression induzieren und die NAMPT-Serumspiegel beeinflussen. Des Weiteren führte eine Behandlung mit Resveratrol in Hepatokarzinomzellen zu einem verminderten SIRT1-Proteinspiegel. Eines der wichtigsten SIRT1-Zielproteine ist das Tumorsuppressorprotein p53. Es wird von SIRT1 direkt gebunden und NAD-abhängig am Lysinrest 382 (K382) deacetyliert. Dies führt zu einer Inhibierung der transkriptionellen Aktivität von p53 und damit zu einer Förderung der Tumorgenese. Unsere Ergebnisse zeigten, dass die durch Resveratrol-Behandlung verminderte SIRT1-Proteinexpression mit einem Anstieg der Acetylierung von p53 (K382) in Hepatokarzinomzellen assoziiert war. Die daraus resultierende verstärkte transkriptionelle Aktivität von p53 wurde anhand der erhöhten Expression von p21/WAF1/Cip1 nachgewiesen. Diese Ergebnisse belegen, dass Resveratrol die NAMPT- und SIRT1-Proteinfunktion in Hepatokarzinomzellen negativ beeinflusst.

Um festzustellen, ob die Effekte von Resveratrol durch die verminderte NAMPT-Enzymaktivität vermittelt sind, wurden die Zellen mit dem NAMPT-Enzymprodukt NMN ko-stimuliert. Die Ergebnisse zeigten jedoch, dass NMN keine protektive Wirkung auf die Resveratrol-vermittelten Effekte, wie Zellzyklusarrest und Apoptose, ausübte. Dies macht deutlich, dass die Verfügbarkeit von NMN und NAD nicht den entscheidenden Faktor in der Wirkungsweise von Resveratrol darstellt. Mittels SIRT1-Überexpression sollte anschließend herausgefunden werden, ob die Effekte von Resveratrol durch eine verminderte SIRT1-Funktion in Hepatokarzinomzellen vermittelt wurden. Doch auch eine SIRT1-Überexpression konnte keinen protektiven Effekt auf die Resveratrol-induzierte Apoptose in Hepatokarzinomzellen ausüben. Jedoch konnten die Ergebnisse zeigen, dass eine verstärkte SIRT1-Expression den Resveratrol-induzierten Zellzyklusarrest sowie die p53-Hyperacetylierung reduzieren konnte. Es ist daher anzunehmen, dass SIRT1 eine Rolle bei dem p53-vermittelten Zellzyklusarrest, aber nicht bei der Resveratrol-vermittelten Apoptose, spielt. Zudem konnte die Resveratrol-induzierte NAMPT-Freisetzung durch eine SIRT1-Überexpression aufgehoben werden, was auf einen Zusammenhang zwischen der SIRT1-Aktivität und der Regulation der NAMPT-Sekretion hindeutet. Insgesamt konnten die Ergebnisse dieser experimentellen Studie zeigen, dass Resveratrol die

NAMPT-vermittelte NAD-Biosynthese in humanen Hepatozyten verstärkte und darauf aufbauend die Aktivität von SIRT1 positiv beeinflussen könnte. Hingegen führte die Behandlung von Hepatokarzinomzellen mit Resveratrol zu einer verminderten NAMPT- und SIRT1-Proteinfunktion, was mit einem p53-vermittelten Zellzyklusarrest assoziert war. Jedoch wurde festgestellt, dass weder NAMPT noch SIRT1 an der Apoptose-induzierenden Wirkung von Resveratrol beteiligt sind. Vielmehr könnte eine kollektive Wirkung verschiedener Signalwege für die Resveratrol-vermittelte Apoptose-Induktion in Krebszellen verantwortlich sein.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass eine FK866-vermittelte NAMPT-Inhibierung zu einer Reduktion der Viabilität von Hepatokarzinomzellen führt und die NAMPT-vermittelte NAD-Biosynthese eine bedeutende Rolle im Energiestoffwechsel von Krebszellen spielt. Zudem belegt diese Arbeit, dass FK866 in Hepatokarzinomzellen eine deutlich höhere chemotherapeutische Wirksamkeit besitzt als in normalen Hepatozyten, was unter anderem auf eine erhöhte basale NAMPT-Aktivität in Hepatokarzinomzellen zurückzuführen ist. Resveratrol bewirkt in Hepatokarzinomzellen, im Gegensatz zu normalen primären Hepatozyten, eine verminderte Aktivität von NAMPT und SIRT1, was mit einem Zellzyklusarrest in der S- und G2/M-Phase assoziiert ist.

Diese Ergebnisse tragen zu einem besseren Verständnis der molekularen Wirkungsweise von FK866 und Resveratrol in Hepatokarzinomzellen bei und identifizieren somit diese Stoffe als interessante potentielle Wirkstoffkandidaten in der Therapie des hepatozellulären Karzinoms.

CHAPTER 1

INTRODUCTION

1.1 Cancer cell metabolism

During the past century cancer research has focused on the altered cellular metabolism and metabolic regulation that are linked to cancer progression. In normal cells, energy transduction leads to the oxidation of nutrients via oxidative phosphorylation. Pyruvate is continuously generated via glycolysis and preferentially transported into mitochondria where it is further metabolized via the tricarboxylic acid cycle (TCA) to generate reduced electron carriers (nicotinamide adenine dinucleotide hydrate (NADH) and flavin adenine dinucleotide dihyrdate ($FADH_2$)). They are reconverted to NAD^+ and FAD by oxidative phosphorylation through the electron-transport chain (complexes I-IV) in the mitochondrial matrix, leading to ATP production by ATP synthase (complex V). In this thesis, the term NAD will be used instead of NAD^+ (oxidized form).

It may not be surprising that tumor cells, in order to meet the increased requirements of proliferation, often display fundamental changes in pathways of energy metabolism and nutrient uptake [1] (**Figure 1**). In 1924, Otto Warburg found that under aerobic conditions, tumor tissue metabolizes approximately tenfold more glucose to lactate in a given time than normal tissue. The so-called Warburg effect describes the glycolytic switch by which cancer cells consume high amounts of glucose for aerobic glycolysis, but only a small portion for oxidative phosphorylation (respiration) even in the presence of sufficient oxygen [2]. It is still a matter of debate why cancer cells switch to less-efficient ATP generation from aerobic glycolysis rather than from oxidative phosphorylation in mitochondria. However, one explanation is that increased glycolysis leads to elevated glycolytic intermediates into intersecting biosynthetic pathways, generating amino acids and nucleosides required for building macromolecules [3]. Work in recent years has revealed that the glycolytic switch is promoted by oncogenes and inhibited by tumor suppressors, indicating that it is intrinsically associated with oncogenic transformation [4, 5]. However,

normal cells also undergo a glycolytic switch and other metabolic changes to proliferate, such as activated T lymphocytes [6].

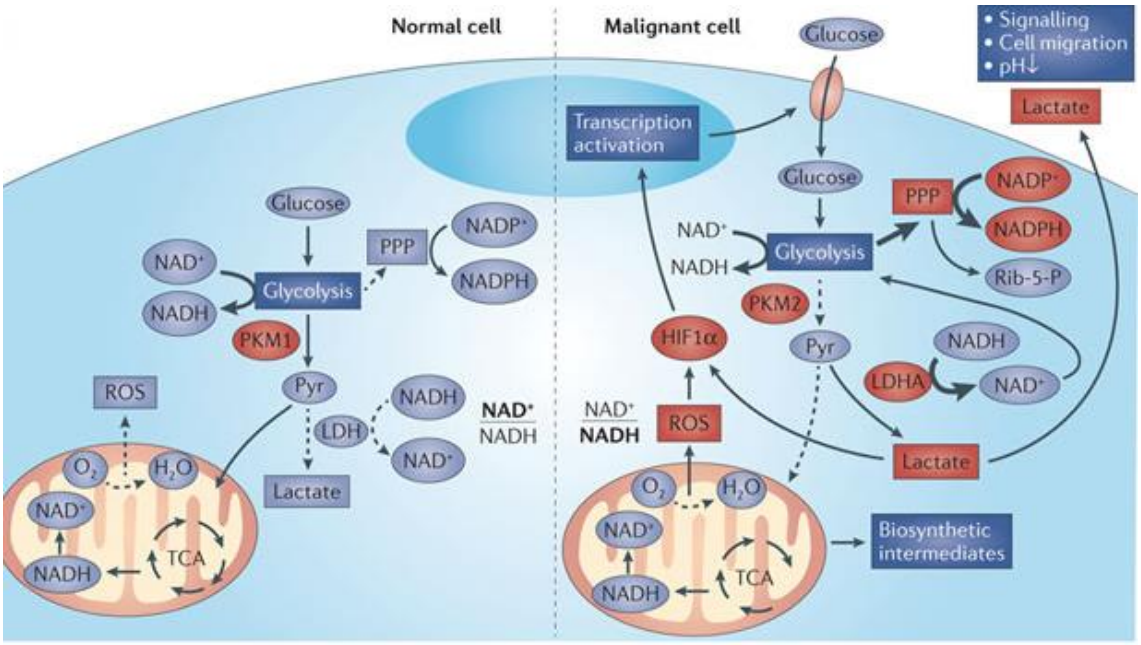

Figure 1. Cancer cell metabolism (the Warburg effect). Comparison of cell metabolism in normal cells (left side) and malignant cells (right side). Normal cells prefer the oxidation of nutrients via oxidative phosphorylation. The glycolysis continuously generates pyruvate (Pyr) which is preferentially transported to the mitochondria and metabolized via the tricarboxylic acid (TCA) cycle. In cancer cells, even under normoxic conditions, a high rate of glycolysis and glucose uptake is observed while respiration remains almost unchanged (aerobic glycolysis). The lactate dehydrogenase isoform A (LDHA) in tumor cells converts accumulated pyruvate to lactate, thereby regenerating NAD from NADH for glycolysis. Excess lactate is secreted and contributes to an extracellular environment that promotes tumor progression. Glycolytic intermediates are mostly diverted into the pentose phosphate pathway (PPP). As a result, NADPH is produced to counteract oxidative stress and to build up macromolecules including the production of ribose-5-phosphate (Rib-5-P) for nucleic acid synthesis. Hypoxia inducible factor 1α (HIF-1α) supports the glycolytic flux and tumor survival. Other abbreviations: PKM1 and 2 (Pyruvate kinase 1 and 2), ROS (reactive oxygen species). [7]

In recent years, meta-analysis of published microarray data sets uncovered metabolic pathways that are commonly up-regulated across tumor types, namely glycolysis, nucleotide synthesis and the pentose phosphate pathway [8]. It has also become clear that the cancer specific alterations in signaling pathways are affected by mutations and the tumor microenvironment [3, 5]. Furthermore, since the 1950s cancer biologists have also recognized the importance of glutamine as a tumor nutrient. Glutamine is involved in pivotal mechanisms necessary for proliferating tumor cells. For example, it participates in bioenergetics, supports cell defenses against oxidative stress, and complements glucose metabolism in the production of macromolecules [9]. Thus, glutamine shares characteristics with glucose by fulfilling the two important needs of proliferating cancer cells - ATP production and the provision of intermediates for biomass production.

Glycolytic intermediates are also utilized by growing cells to produce fatty acids and non-essential amino acids. Therefore, enhanced glycolysis and *de novo* fatty acid synthesis are indeed characteristic features of cancer cells [10, 11]. This preferred ATP production via increased glycolysis rather than oxidative phosphorylation in cancer cells requires several metabolic alterations, among them increased amounts of the redox co-factors NAD or NAD phosphate (NADP). The lactate dehydrogenase isoform A (LDHA) in tumor cells converts accumulated pyruvate to lactate, thereby regenerating NAD from NADH for glycolysis. Lactate activates the transcription factor hypoxia-inducible factor-1 α (HIF-1α) which stimulates the expression of glycolytic transporters and enzymes supporting high rate of glycolysis [12].

1.2 NAD - a key determinant of cancer cell biology

As the redox couple NAD/NADH, NAD plays a crucial role in numerous cellular processes. Within glycolysis and TCA cycle, NAD functions as electron recipient, whereas in mitochondrial oxidative phosphorylation NADH is essential as an electron donor for the generation of ATP. NAD can also be converted to NADP and reduced to NADPH, which is necessary for fatty acid synthesis and anti-oxidant defense. It is important to note that NADP-dependent processes are linked to the NAD status as NADP synthesis requires NAD. Thus, a depletion of cellular NAD may compromise NADP levels [13].

Besides its role in cellular redox reactions, NAD also serves as substrate for NAD-dependent enzymes, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP-ribosyltransferases (MARTs), ADP-ribosyl cyclases/CD38 and sirtuins [13, 14]. It is therefore one of the most important links between regulatory and bioenergetic processes (**Figure 2**).

NAD-mediated signaling events are involved in the regulation of crucial biological processes, including transcription, cell cycle progression, caloric-restriction responses, apoptosis, circadian rhythms, chromatin dynamics regulation, telomerase activity, intracellular calcium mobilization and metabolic pathways. All these events undergo changes during malignant transformation and cancer progression [15]. Therefore, NAD-dependent metabolic and signaling pathways are altered in cancer cells leading to an increased NAD demand in these cells. The permanent NAD recycling is essential to fuel bioenergetic processes and maintain cell proliferation. NAD-dependent enzymes release nicotinamide during their reaction that needs to be recycled to maintain tissue NAD levels [16]. Most NAD in humans is resynthesized from nicotinamide [17]. The conversion of

nicotinamide to NAD is mediated by nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the mammalian NAD biosynthesis starting from nicotinamide [16, 18].

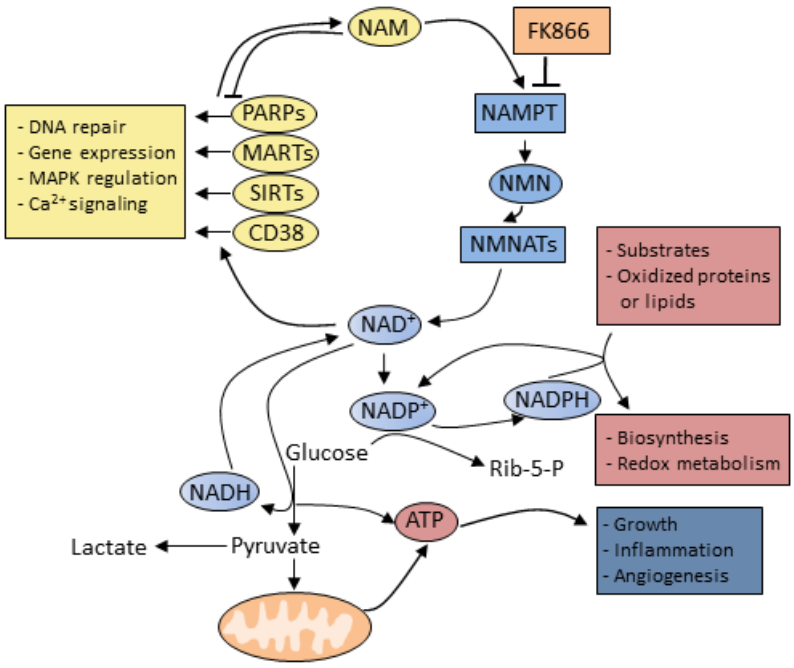

Figure 2. NAD - key regulator in cancer cell metabolism. The function of NAD as coenzyme is depicted in light blue, such as in glycolysis and the pentose phosphate pathway. NAD is also consumed by NAD-dependent enzymes shown in yellow, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs), sirtuins (SIRTs), ADP-ribosyl cyclases (CD38) with a concomitant release of nicotinamide (NAM) which has a negative feedback function. NAM enters the NAD salvage pathway (blue) and is recycled by nicotinamide phosphoribosyltransferase (NAMPT) to nicotinamide mononucleotide (NMN) and further to NAD by nicotinamide mononucleotide adenylyltransferases (NMNATs). The NAMPT inhibitor FK866 is depicted in orange.

A detailed overview about the physiological and pathophysiological functions of NAMPT is given in **Chapter 2**. An inhibition of NAMPT activity represents one of the most powerful ways to lower organismal NAD levels. Alternative NAD precursors, such as nicotinic acid (NA), nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN) can be administered to rescue the toxic effects of NAMPT inhibition [19]. This strategy could be used to increase the therapeutic potential of NAMPT inhibitors through the rescue of normal tissues.

1.3 The NAD-dependent deacetylase SIRT1 and cancer

Cancer cells possess a higher activity of NAD-dependent enzymes than non-transformed cells. NAD-dependent enzymes are involved in many crucial cellular pathways of cancer

cell progression [7]. For example, PARP1 is responsible for DNA repair and therefore necessary for DNA integrity [20]. Activation of PARP1 consumes large amounts of NAD [21] and a lot of research interest has been focused on PARP inhibition in cancer therapy [22].

Another important family of NAD-dependent enzymes are sirtuins, which belong to the class III of histone deacetylases (HDACs), also known as lysine deacetylases. Sirtuins can deacetylate not only histones, but also non-histone proteins at specific loci and thus directly link metabolic and bioenergetic homeostasis and gene regulation. Furthermore, NAD-dependent deacetylation regulates the activity, stability or localization of target proteins. First discovered as caloric restriction response proteins who extend life span in lower organisms, further studies revealed that sirtuins are involved in a multitude of biological processes, such as senescence, aging, DNA repair, survival, metabolism and proliferation. Some members of the various classes of histone deacetylases (HDACs) have been shown to be overexpressed in diverse cancers. Thus, resulting changes in the acetylation patterns on proteins may contribute to cellular transformation and tumor progression. Accumulating evidence has indicated that SIRT1, the most studied of the group of SIRTs, is a key regulator of life span extension, DNA damage, metabolism, stress, inflammation, and cancer development [23–28]. The non-histone deacetylation targets of SIRT1 include transcription factors, such as p53, Forkhead box 'Other' (FoxO) proteins, hypoxia-inducible factor 2α (HIF-2α), E2F1, hypermethylated in cancer 1 (HIC1), B-cell lymphoma 6 protein (BCL6), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), peroxisome proliferator-activated receptor γ (PPARγ), nuclear factor κB (NF-κB) and others; as well as DNA repair proteins including Ku-70 and signaling factors such as endothelial nitric oxide synthase (eNOS) [27, 29].

SIRT1 has been described to act as tumor promotor as well as tumor suppressor [23, 27]. Influencing diverse physiological processes, it is not surprising that the role of SIRT1 in cellular growth control is complex and its enzymatic activity exerts important cell-type specific effects [30, 31]. SIRT1 overexpression has been shown in different cancer types, such as hematopoietic malignancies like leukemia and several solid tumors of the breast, colon, prostate and liver [32–38]. Due to the fact that SIRT1 activity influences diverse physiological processes associated with cell growth and motility [26, 39, 40], its overexpression could have grave consequences for tumor progression. In contrast, SIRT1 inhibition was shown to be effective in reducing cell proliferation, while inducing apoptosis in cancer cells [41–43]. The specific small-molecule inhibitor of SIRT1 catalytic activity, EX-527 increased p53 acetylation at lysine 382 but had no effect on viability of primary

human mammary epithelial cells [44]. However, tumor cells treated with EX527 showed increased cell cycle arrest [43]. Additionally, SIRT1 plays also an important role in DNA damage response processes which are crucial for maintaining genome stability when cells are exposed to genotoxic factors [28, 45].

The first discovered non-histone target of SIRT1, p53, is suggested to play a central role in SIRT1-mediated functions in tumorigenesis and senescence [46, 47]. Normally, p53 protein is not activated and thus maintained at a low level through the MDM2-mediated ubiquitination and degradation pathway. However, under cellular stress including genotoxic stress, p53 protein rapidly accumulates and activates downstream biological functions, including cell cycle arrest, DNA repair, and apoptosis [48, 49]. Mutations within the p53 gene have been documented in more than half of all human tumor types [50]. Post-translational modifications, such as phosphorylation, ubiquitination and acetylation regulate the amount, stability and activity of p53. The C-terminal region acetylation of p53 activates its sequence-specific DNA binding activity and target gene expression as well as increases its stability due to inhibition of ubiquitination at acetylated lysines [48]. SIRT1 deacetylates p53 at lysine residue 382 (K382) and thereby inhibits its transcriptional activity and apoptosis [47]. For example, SIRT1 knockout mice showed increased p53 acetylation and were more susceptible to radiation-induced apoptosis, raising the possibility that SIRT1 can facilitate tumor growth by antagonism of p53 [51] and inhibition of SIRT1 activity induces senescence-like cell growth arrest in human cancer cells [42, 43, 52].

1.4 Targeting the NAMPT-mediated NAD salvage pathway may prove a cancers Achilles' heel

The uncontrolled proliferation presents a significant bioenergetic challenge to cancer cells as they must generate enough energy and acquire or synthesize biomolecules at a sufficient rate to meet the demands of proliferation. As mentioned above, targeting the NAD salvage pathway in cancer cells represents one of the most powerful tools to disturb the cancer cell energy metabolism and to modify NAD-dependent signaling pathways associated with DNA repair, proliferation, cell cycle and apoptosis.

NAMPT inhibition

Due to increased activity of NAD-dependent enzymes cancer cells rely on continuous supply of NAD from nicotinamide. Therefore, cancer cells often maintain intracellular NAD levels by overexpressing NAMPT, the key enzyme in the NAD-salvage pathway

starting from nicotinamide. NAMPT overexpression has been shown in different cancer types, including colorectal, ovarian, breast, gastric, prostrate, well-differentiated thyroid, and endometrial carcinomas, and myeloma, melanoma, and astrocytomas [53]. However, there are currently no studies available about the expression and regulation of NAMPT in hepatocellular carcinoma (HCC). HCC belongs to the second leading cause of cancer-related deaths [54]. The only available proven systemic therapy for HCC is the multi-target kinase inhibitor sorafenib. An effective second-line agent for those with sorafenib failure or intolerance has yet to be identified [55]. Thus, there is an ongoing search for molecular pathways and novel compounds as potential treatment option for HCC.

Clinical studies reported increased serum NAMPT levels in cancer patients with a positive correlation between either tissues or circulating levels and stage progression. *In vitro* studies could show that cell lines overexpressing NAMPT were significantly more resistant to chemotherapeutic agents than control cells. In contrast, stable NAMPT knock-down cells were shown to be more sensitive to methylmethane sulfonate (MMS), a DNA alkylating agent [56]. Additionally, NAMPT was shown to activate the mitogen-activated protein kinases (MAPK)-dependent pathway and to stimulate the vascular endothelial growth (VEGF) factor promoting angiogenesis, a crucial process during tumor growth and expansion [57, 58]. Targeting NAMPT activity represents a novel therapeutic strategy for HCC.

The specific NAMPT inhibitor FK866 (also known as APO866 or WK175) is a competitive inhibitor and has no inhibitory effect on nicotinic acid phosphoribosyltransferase (NAPRT) or nicotinamide mononucleotide adenylyltransferase (NMNAT) [59–61]. Crystal structure analysis revealed that FK866 is bound in a tunnel at the interface of the NAMPT dimer, and mutations in this binding site can abolish the inhibition by FK866. Thus, FK866 competes with nicotinamide at the active binding site of NAMPT. Given that NAMPT is the sole enzyme that recycles nicotinamide to NAD, NAMPT inhibition not only affects NAD levels but may also influence nicotinamide levels. Nicotinamide, which non-competitively inhibits sirtuins without affecting their binding of NAD, has been widely used as a pan-sirtuin inhibitor [62, 63]. FK866-mediated NAMPT inhibition causes a delayed cell death by ATP depletion [59, 64]. The mechanism of cancer cell death is suggested to be apoptosis, depending on cellular ATP levels [59, 65, 66]. However, autophagy has been also reported [67]. In recent years, FK866 has been evaluated in a broad variety of tumors, including solid tumor-derived cells and leukemia cells *in vitro* [68, 69] and in nude mouse xenografts, where FK866 was able to reduce or

attenuate tumor growth [69, 70]. In healthy cells, FK866 showed low cytotoxicity due to their comparatively slow metabolic rate.

Currently, FK866 is undergoing phase II clinical trials for advanced melanoma and cutaneous T-cell lymphoma (ClinicalTrials.gov Identifier: NCT00431912), for phase I/II for refractory B-cell Chronic Lymphocytic Leukemia (ClinicalTrials.gov Identifier: NCT00435084) as well as advanced cutaneous melanoma (ClinicalTrials.gov Identifier: NCT00432107). In pre-clinical studies thrombocytopenia was the dose limiting toxicity and no other hematologic toxicities were noted other than mild lymphopenia and anemia [61].

Resveratrol and cancer

Increasing research efforts have been made to investigate the use of natural agents, alone or in combination with established anti-cancer agents for cancer therapy. During the past decade, significant work has been done towards identifying the health-beneficial effects of the antioxidant resveratrol in a variety of bioassay- and disease- models. The polyphenol resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring phytoalexin found in grapes, berries and peanuts. It is synthesized as a plant antibiotic to counteract pathogen infections, including fungal infection or UV radiation and in response to environmental and mechanic stress [71, 72].

Since studies have shown that resveratrol is able to partially mimic the effects of a calorie restriction [73, 74] which is known to extend lifespan in diverse species and increase NAMPT and SIRT1 expression, further studies identified resveratrol as potential natural SIRT1 activator [75]. Whether or not SIRT1 is directly activated by resveratrol has been the subject of a contentious debate [75–77]. Recent studies reported that resveratrol action only triggers SIRT1-mediated deacetylation in a substrate-specific fashion, and even led to a paradoxical inhibition of SIRT1 actions on certain substrates. Many contradicting studies called resveratrol as a SIRT1 activator into question. The assay that initially identified resveratrol as a SIRT1 activator has used the non-physiological "Fluor de Lys" substrate for screening which can lead to artefactual results. Thus, resveratrol failed to activate SIRT1 with native peptides or full-length protein substrates, whereas resveratrol does activate SIRT1 when using fluorophore-containing peptides [76–79]. There are also controversies between the *in vivo* effects of resveratrol treatment and SIRT1 genetic gain-of-function models [80]. Altogether, these data indicate that resveratrol can no longer be seen as global SIRT1 activator. Further, one has to take into account that there is

uncertainty whether resveratrol itself or one of its metabolized forms accounts for the biological effects.

Recent studies revealed that resveratrol activates the AMP-activated kinase (AMPK) whereas the underlying mechanism of activation is still unclear. One explanation could be the decrease of ATP levels and concomitant increase in AMP/ATP or ADP/ATP ratios induced by high concentrations of resveratrol (100-300μM) [81]. Low concentration of resveratrol (< 50μM) can also increase AMPK activation without decreasing energy equivalents [82, 83] but by inhibition of phosphodiesterases (PDE) that leads to increased cAMP levels [84] or SIRT1 activation [85]. Moderate dose of resveratrol has been shown to require SIRT1 activity to activate AMPK and to exert beneficial effects on mitochondrial function while high dose of resveratrol activated AMPK in a SIRT1-independent manner, demonstrating that resveratrol dosage is a critical factor.

Many preclinical and clinical studies have shown that resveratrol can prevent or slow the progression of a wide variety of age-associated diseases, including cancer, diabetes, arthritis, and coronary, neurodegenerative, and pulmonary diseases [86]. Resveratrol was shown to inhibit carcinogenesis at all three stages (initiation, promotion, and progression). The same study demonstrated that topical application of resveratrol reduced the number of skin tumors in a mouse melanoma model by up to 98% [87]. Systemic administration of resveratrol has since been shown to inhibit the initiation and growth of tumors in a wide variety of rodent cancer models [86]. Several *in vitro* studies reported that resveratrol exerts its multiple anti-cancer effects by inducing cell cycle arrest and apoptosis [88–90]. For example, resveratrol prevents the expression of the tumor-derived nitric oxide synthase to block tumor growth and migration and is also acting as an antioxidant to prevent DNA damage that can lead to tumor formation [88, 89, 91]. However, on the other hand recent studies also revealed that resveratrol induces DNA damage in colorectal cancer as well as in head and neck squamous carcinoma [92, 93] which puts the antioxidant properties of resveratrol into question. In addition, the activity of cyclooxygenases (COX) which are known to play a role in tumorigenesis by promoting tumor cell proliferation, are also inhibited by resveratrol [94, 95].

In recent years, numerous studies further revealed that resveratrol decreases the DNA binding activity of the NF-κB, a transcription factor that is known to be upregulated in cancers and induces the transcription of genes that promote tumor growth [96–98]. Likewise, several reports demonstrated the chemopreventive and chemotherapeutic potential of resveratrol in HCC. For example, it was found that resveratrol prevents

diethylnitrosamine (DENA)-initiated hepatocarcinogenesis in rats through suppression of inflammation and oxidative stress [99]. The effects of resveratrol on NAMPT and SIRT1 in hepatocarcinoma cells have yet to be investigated. Elucidating how resveratrol exerts its effects would provide not only new insights in its fundamental biological actions but also open new avenues for the design and development of more potent drugs for HCC therapy.

1.5 Metabolic Checkpoints: AMPK and mTOR

AMPK

The AMPK is a highly conserved heterotrimeric serine/threonine kinase and occupies a central role in linking metabolism and cancer development [100, 101]. Its pharmacological activation by metformin was used for the treatment of metabolic syndrome and type 2 diabetes. During the last decades, there is emerging evidence showing that AMPK is implicated in cancer cell growth and metabolism [100-102]. Likewise, under conditions of metabolic stress, typically by an increase in the cellular AMP/ATP ratio under conditions such as glucose deprivation, hypoxia, ischemia, and heat shock, AMPK is activated [103–105]. One of the newly uncovered links directly connecting cell metabolism and cancer came from the discovery that the serine/threonine kinase LKB1 (liver kinase B1; also known as serine/threonine kinase 11 - STK11), a known tumor suppressor, was the key upstream activator of AMPK [106, 107]. Once activated it regulates various processes, including development, cell cycle progression, apoptosis, and autophagy [108]. Inactivation of AMPK has been implicated in cancer development and malignant behaviors in several cancers, including prostate, lung, and breast. Loss of AMPKα increased HIF-1α expression, thereby promoting the Warburg effect by increasing aerobic glycolysis and lactate production [101]. A very recent paper showed that AMPK was less activated in the majority of the patients with HCC and discussed that it may serve as a valuable predicting factor for recurrence and poor survival. Moreover, activation of AMPK by metformin inhibited NF-κB and signal transducer and activator of transcription 3 (STAT3)-signaling activity, and thus inhibited HCC growth [109]. On the contrary, another study reported that AMPK promotes hepatocarcinogenesis by destabilizing p53 in a SIRT1-dependent manner [110]. Thus, modulation of AMPK has emerged as an important target for the treatment of obesity, diabetes, and cancer.

mTOR

Under energy starvation condition it was shown that AMPK phosphorylates tuberous sclerosis complex 2 (TSC2) to inhibit mammalian target of rapamycin (mTOR) signaling [111]. This observation revealed a direct connection of AMPK with mTOR and cancer. mTOR is a highly conserved serine/threonine kinase involved in regulating major cellular functions including growth and proliferation. mTOR functions as the catalytic subunit of two distinct protein kinase complexes, designated as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), respectively [112]. Deregulation of the mTOR signaling pathway is one of the most commonly observed pathological alterations in human cancers. Components of this complex are often overexpressed in a large number of cancers and promote malignant transformation in experimental systems [113].

Specifically, mTORC1 is regulated by sensing intracellular as well as extracellular stimuli, such as stress, nutrients, energy, oxygen levels and growth factors. Once activated mTORC1 regulates protein translation and cell metabolism through directly phosphorylating the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase (p70S6K). It also activates sterol regulatory element-binding protein (SREBP) and autophagy components to promote protein and lipid synthesis, lysosome biogenesis, energy metabolism and to inhibit autophagy [113]. In contrast, mTORC2 is less sensitive to nutrients but more sensitive to growth factors. Studies on AMPKα-deficient tumors demonstrated an increased activation of the mTORC1 targets p70S6K and 4E-BP1 suggesting that AMPK and mTOR serve as a signaling nexus for regulating cellular metabolism, energy homeostasis and cell growth. Thus, a dysregulation of each pathway may contribute to cancer development. In recent years, many studies revealed a link between the NAMPT-mediated NAD metabolism and regulation of mTOR signaling. Recent studies showed that p70S6K is regulated by sirtuins-mediated acetylation that influences the mTORC1-dependent p70S6K activation [114] and NAMPT overexpression reduces mTOR activation [115].

1.6 Aims of this thesis

As mentioned above, cancer cell metabolism is characterized by fundamental changes in pathways of energy metabolism. These metabolic alterations require increased amounts of NAD. Thus, cancer cells have an increased NAD turnover and elevated activity of NAD-dependent enzymes, such as sirtuins. The chemopreventive and chemotherapeutic potential of the SIRT1 modulator resveratrol was shown in animal models of HCC. The

AMPK/mTOR signaling network is connected to and influenced by sirtuins and the NAD/NADH ratio and was also shown to be involved in hepatocarcinogenesis.

Based on these facts, I hypothesised that targeting NAMPT activity by pharmacological inhibition reduces cell viability of hepatocarcinoma cells. Thus, the aims of my presented work were **(1)** to investigate the chemotherapeutic potential and molecular mechanisms of the specific NAMPT inhibitor FK866 and the phytochemical resveratrol on hepatocarcinoma cells and to find out whether there are differences compared to non-cancerous primary human hepatocytes; **(2)** to investigate whether NAMPT inhibition by FK866 would induce changes in the energy metabolism and the AMPK/mTOR signaling that would lead to a reduction of cancer cell proliferation and **(3)** to address the relevance of NAMPT and SIRT1 in resveratrol´s mode of action as well as the underlying mechanism of resveratrol induced cell death in hepatocarcinoma cells.

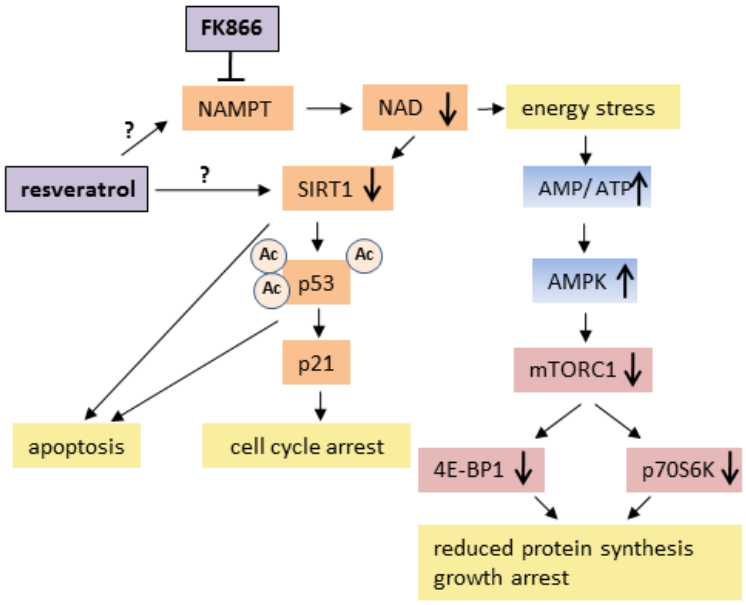

Figure 3. Hypothesis and aims of this thesis. I proposed the following hypothesis: Targeting NAMPT activity by pharmacological inhibition reduces cell viability of hepatocarcinoma cells. To verify my hypothesis I aimed to investigate the chemotherapeutic effects of the NAMPT inhibitor FK866 and the phytochemical resveratrol. I intended to find out whether a FK866-mediated NAMPT inhibition would induce energy stress in hepatocarcinoma cells that would lead to activation of AMP-activated kinase (AMPK) and downregulation of mammalian target of rapamycin (mTOR) complex1 (mTORC1)-signaling. If NAD depletion would activate AMPK, FK866 would be identified as novel AMPK activator in hepatocarcinoma cells. I also aimed to analyse the different sensitivities of hepatocarcinoma cells and non-cancerous hepatocytes to NAMPT inhibition. Reduced NAD levels would negatively influence SIRT1 deacetylase activity which was described to increase p53 acetylation at lysine residue 382 and to induce cell cycle arrest via induction of p21. Because resveratrol has been described as natural SIRT1 modulator, I aimed to investigate the relevance of NAMPT and SIRT1 in resveratrol´s mode of action and chemotherapeutic function. I assumed that resveratrol does not act as a SIRT1 activator in cancer cells. Thus, it should be addressed whether NAMPT and SIRT1 are involved in resveratrol-mediated effects on cell cycle arrest or apoptosis.

Therefore, I used different models of human hepatocarcinoma cells and performed a comprehensive study covering aspects in the field of cellular responses, enzyme functions and signaling pathways. As control, non-cancerous primary human hepatocytes were used. These investigations provide new insights into the role of the NAMPT-mediated NAD salvage pathway in energy metabolism of hepatocarcinoma cells and contribute to a better understanding of the molecular mechanisms and chemotherapeutic function of FK866 and resveratrol and will help to identify new therapeutic strategies for HCC cancer therapy.

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CHAPTER 2

Physiological and pathophysiological roles of NAMPT and NAD metabolism

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Nicotinamide phosphoribosyltransferase (NAMPT) is a regulator of the intracellular NAD (nicotinamide adenine dinucleotide) pool. NAD is an essential coenzyme involved in cellular redox reactions and a substrate for NAD-dependent enzymes. NAD concentrations are decreased in various metabolic disorders and during aging. Through its NAD-biosynthetic activity, NAMPT was shown to influence the activity of NAD-dependent enzymes, thereby regulating cellular metabolism. Besides its intracellular enzymatic function, NAMPT is also found in circulation and has cytokine-like activity. Extracellular NAMPT levels were shown to be associated with various metabolic disorders. NAMPT is able to modulate important processes in the pathogenesis of obesity and related disorders like non-alcoholic fatty liver disease and type 2 diabetes by regulating the oxidative stress response, apoptosis, lipid and glucose metabolism, inflammation and insulin resistance. NAMPT also plays a crucial role in cancer cell metabolism, is often overexpressed in tumor tissues and an experimental target for anti-tumor therapies. We will discuss the current understanding of NAMPT functions with an emphasis on recent progress on NAMPT's physiological role and its relevance in various human diseases and conditions, such as obesity, non-alcoholic fatty liver disease, type 2 diabetes, aging and cancer.

2.1 Introduction: NAD metabolism, NAMPT and mechanism of action

Nicotinamide phosphoribosyltransferase (NAMPT) was originally discovered as a cytokine named Pre-B-cell colony enhancing factor (PBEF) [1] acting as a co-factor for B-cell maturation. The bacterial and murine homologs of NAMPT were later identified as enzymes involved in NAD biosynthesis [2, 3]. Additionally, NAMPT was described to be highly expressed in visceral compared to subcutaneous adipose tissue and its plasma levels correlated with obesity outcome. Therefore, NAMPT was recognized as an adipokine and renamed visfatin [4]. NAMPT/visfatin was described to directly bind to and activate the insulin receptor at a binding site distinct from insulin. However, due to controversies about the results, this paper was retracted [5]. While all three names (NAMPT, PBEF, visfatin) have been used, "NAMPT" has been approved as the official nomenclature of the protein and the gene by both the HUGO Gene Nomenclature Committee and the Mouse Genomic Nomenclature Committee [6]. NAMPT will therefore be used throughout this review. We will discuss the current understanding of NAMPT functions with an emphasis on recent progress on NAMPT's physiological role and its relevance in various human diseases and conditions, such as obesity, non-alcoholicfatty liver disease, type 2 diabetes, aging and cancer.

NAD biosynthesis and NAMPT enzyme activity

NAD (nicotinamide adenine dinucleotide) is an essential coenzyme involved in cellular redox reactions and a substrate for NAD-dependent enzymes. In mammals, NAD can be synthesized either from tryptophan or from vitamin B3, which includes nicotinamide, nicotinic acid and nicotinamide riboside. The *de novo* pathway using L-tryptophan mainly occurs in the liver, but it is assumed that the main source of NAD is produced by salvage pathways using vitamin B3 [7]. That includes the NAD precursors nicotinamide (NAM), nicotinic acid (NA) and nicotinamide riboside (NR). Nicotinamide rather than nicotinic acid is thought to be the predominant NAD precursor in mammals [8–10]. Nicotinamide is also a product of deacetylation and ADP-ribosylation reactions catalysed by NAD-dependent enzymes [11–13]. NAMPT produces NMN (nicotinamide mononucleotide) from nicotinamide and PRPP (5'-phosphoribosyl-1-pyrophosphate), catalysing the rate-limiting step in the mammalian NAD salvage pathway from nicotinamide [3, 14]. NMN, together with ATP, is then converted into NAD by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT). NR is converted into NMN by

nicotinamide riboside kinase (NRK) [15], entering the NAD salvage pathway. A simplified overview of mammalian NAD metabolism is depicted in **figure 1**.

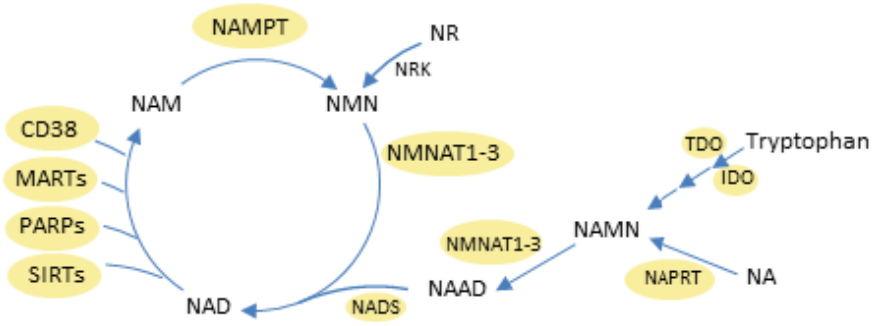

Figure 1. Overview of the mammalian NAD metabolism. The NAD *de novo* kynurenine pathway is comprised of several steps. The first rate-limiting step is catalysed by tryptophan-2,3,-dioxigenase (TDO) or indoleamine-2,3,-dioxigenase (IDO). By multiple reactions, L-tryptophan is converted to nicotinic acid mononucleotide (NAAM). At this stage, nicotinic acid (NA) enters the pathway and is phosphoribosylated by nicotinic acid phosphoribosyltransferase (NAPRT). NAAM is then converted to nicotinic acid adenine dinucleotide (NAAD) by nicotinamide/nicotinic acid mononucleotide adenylyltransferase enzymes 1-3 (NMNAT1-3). Finally, NAAD is amidated by NAD synthase (NADS) to yield NAD. Nicotinamide (NAM) is another NAD precursor and a product of deacetylation and ADP-ribosylation reactions catalysed by NAD-dependent enzymes such as sirtuins (SIRTs), poly (ADP-ribosyl) transferases (PARPs), mono (ADP-ribosyl) transferases (MARTs) and ADP-ribosyl cyclases like CD38. Nicotinamide phosphoribosyltransferase (NAMPT) produces nicotinamide mononucleotide (NMN) from nicotinamide and 5'-phosphoribosyl-1-pyrophosphate (PRPP), catalysing the rate-limiting step in the NAD salvage pathway from nicotinamide. NMN is then converted into NAD by NMNAT1-3. Nicotinamide riboside (NR) enters the NAD biosynthesis pathway by phosphorylation to NMN catalysed by nicotinamide riboside kinase (NRK).

NAMPT occurs intracellular, mainly located in the cytoplasm and nucleus, but is also found in mitochondria [16], and in circulation [17]. Significant NAMPT sequence homology exists among prokaryotic organisms, primitive metazoans such as marine sponges, and humans [2], suggesting a crucial role for NAMPT in cellular metabolism and survival. Several groups have characterized the structural and enzymological features of mammalian NAMPT [18–25]. Structural and mutagenesis studies have shown that mutants with impaired dimerization exhibit attenuated enzymatic activity [20] and that Asp219 is important in defining the substrate specificity of NAMPT [23], which does not convert nicotinic acid [14, 26]. NAMPT can autophosphorylate at H247 [18, 20] which increases its affinity for nicotinamide and its enzymatic activity up to 1000-fold [18]. To our knowledge, no other regulatory post-translational modification has been described so far. However, this does not preclude a role for phosphorylation, ubiquitinylation and acetylation in the regulation of NAMPT function.

NAMPT single nucleotide polymorphisms (SNPs) and regulation of NAMPT expression and activity

From analysing tissue sources of 719 cDNA clones, it was found that NAMPT is expressed in nearly all organs, tissues, and cells examined [1, 27]. This ubiquitous distribution of NAMPT suggests pleiotropic functions of this protein in human physiology. A number of single nucleotide polymorphisms (SNPs) have been detected and are associated with a number of disease phenotypes (e.g. acute respiratory distress syndrome [28], coronary artery disease [29], obesity [30], acute lung injury [31, 32], sepsis [33], bladder cancer [34], glucose and lipid parameters [35]). However, several studies found that genetic variations in the NAMPT gene do not play a major role in the development of obesity or type 2 diabetes[36–38]. The relevance for genetic variations of NAMPT in disease development may depend on whether the active site, the assembly of the active dimer form or gene expression is affected. A recent study showed that rs2302559 significantly correlates with NAMPT serum levels [39]. Therefore, functional association studies of NAMPT variants and their secretion patterns are yet to be performed.

Interestingly, previous studies could show that the *NAMPT* gene is regulated by the core clock machinery. Intracellular NAD levels cycle within a 24-hour rhythm. CLOCK: BMAL1 regulates the circadian expression of NAMPT. Sirtuin1 (SIRT1) is recruited to the *NAMPT* promoter and contributes to the circadian synthesis of NAMPT [40, 41]. Since obesity influences the expression of circadian regulatory genes like *Clock*, *Rev-Erb-a* and *Bmal* [42], changes in NAMPT expression and function might contribute to the development of obesity-related co-morbidities. Furthermore, hepatic forkhead box protein (FoxO) transcription factors regulate *NAMPT* gene expression and the hepatic triglyceride homeostasis. For instance, NAMPT overexpression reduced, whereas its knockdown increased hepatic triglyceride levels *in vitro* and *in vivo* [43]. Studies on human NAMPT in skeletal muscle reported that glucose restriction and exercise increased NAMPT expression and that mitochondria content correlated with NAMPT levels [44]**.** Furthermore, different studies implicated that *NAMPT* gene expression was elevated after stimulation with TNFα [45], IL-6 [46] and under hypoxic conditions [47, 48]. This could be a reason for the increased level of *NAMPT* mRNA in states of obesity and insulin resistance and inflammation. However, contradicting data showed that IL-6 down regulated NAMPT synthesis in adipocytes [49]. Recently, we could demonstrate that stimulation with the plant derived polyphenol resveratrol led to increased *NAMPT* mRNA expression, activity as well as NAD levels in human hepatocytes, although NAMPT protein levels were unchanged. Metabolic factors influencing NAMPT expression are summarised in **table 1**.

Table 1. Metabolic factors influencing NAMPT expression.

Factor	Action
glucose restriction	↑in skeletal muscle [44] ↑in hepatocytes [100] ↑in human preadipocytes and adipocytes [91]
glucose	↑in human adipocytes [87]
fructose	↑in brown and white adipose tissue of fructose-rich-diet fed mice [112] ↓in circulation of fructose-rich-diet fed mice [112]
oxidized low density lipoprotein	↑in 3T3-L1 adipocytes [89]
growth hormone	↓in 3T3-L1 adipocytes [90]
interleukin-6	↑in rheumatoid arthritis [46] ↓in 3T3-L1 adipocytes [90]
Tumor necrosis factor alpha	↑in adipose tissue [45] ↓in 3T3-L1 adipocytes [90]
palmitate	↑in HepG2 cells [103]
leucine	↑in high-fat-diet fed mice [102]
dexamethasone	↑in human preadipocytes and adipocytes [91] ↑in 3T3-L1 adipocytes [90]
isoproterenol	↑in human preadipocytes [91] ↓in 3T3-L1 adipocytes [90]
exercise	↑in skeletal muscle [44]
hypoxia	↑in 3T3-L1 adipocytes [48] ↑in MCF-7 breast cancer cells [47]

↑up and ↓down regulation of NAMPT expression

NAD-dependent enzymes

NAMPT is a regulator of the intracellular NAD pool [3, 14]. Through its NAD-biosynthetic activity, NAMPT was shown to influence the activity of NAD-dependent enzymes, such as sirtuins [14, 50, 16, 51–53] and poly (ADP-ribose) polymerase (PARP)-1 [54], thereby regulating cellular metabolism, mitochondrial biogenesis [55–57] and adaptive stress responses, including inflammatory, oxidative, proteotoxic, and genotoxic stresses [58]. Several comprehensive reviews about the roles of NAD-dependent enzymes in cellular metabolism have been published recently [59–62]. Mono-ADP-ribosyl transferases and ADP- ribosyl cyclases [63] are involved in several physiological processes, including calcium signaling and DNA repair. Since these enzymes also use NAD as substrate and are also inhibited by their product nicotinamide, it is likely that NAMPT action influences their activity.

NAMPT in circulation

Several studies indicate that circulating or extracellular NAMPT (eNAMPT) may function as a growth factor [1, 64–68]. However, the mechanism of NAMPT secretion and its physiological function in extracellular space is far from clear as well as whether NAMPT occurs in the same form and configuration in the cell and in circulation. NAMPT in human serum was described to be secreted from adipocytes [17, 69]. During the last years, many other cell types have been identified to release NAMPT, such as hepatocytes [70, 71], leukocytes [27], cardiomyocytes [72], neurons [73, 74], amniotic epithelial cells [75] and LPS-activated monocytes [76, 77]. Whether circulating NAMPT is enzymatically active *in vivo* or not is still a matter of debate due to the low concentration of PRPP and ATP in extracellular space [78]. However, it raises the question whether enzymatic activity of circulating NAMPT is linked to pathophysiological conditions where plasma PRPP and ATP levels may increase due to cell death. Since a receptor for eNAMPT has not been discovered yet, the mechanism of NAMPT signal transduction continues to be subject of scientific research as it is of major importance in targeting circulating NAMPT in various pathological conditions. Whether circulating NAMPT exhibits pro- or anti-inflammatory activity is still being debated [17, 72–74, 79, 80]. However, several studies have shown pro-inflammatory effects of eNAMPT on different cell types which involve iNOS induction [81], ERK1/2 activation [65], NF-$\varkappa$B activation [33, 81] and cytokine production, such as TNFα, IL-6, IL-1β [33] and TGF-β [82]. **Figure 2** gives an overview on NAMPT physiological actions. It appears that inflammatory cytokine production and *NAMPT* gene expression are regulated by a positive feedback activation loop and that NAMPT acts as a stress-response protein. This implicates an interesting connection between chronic inflammation and NAMPT-mediated NAD biosynthesis. A better understanding of its mechanisms of action is a prerequisite for the use of NAMPT-related pathways as a therapeutic option in relevant diseases.

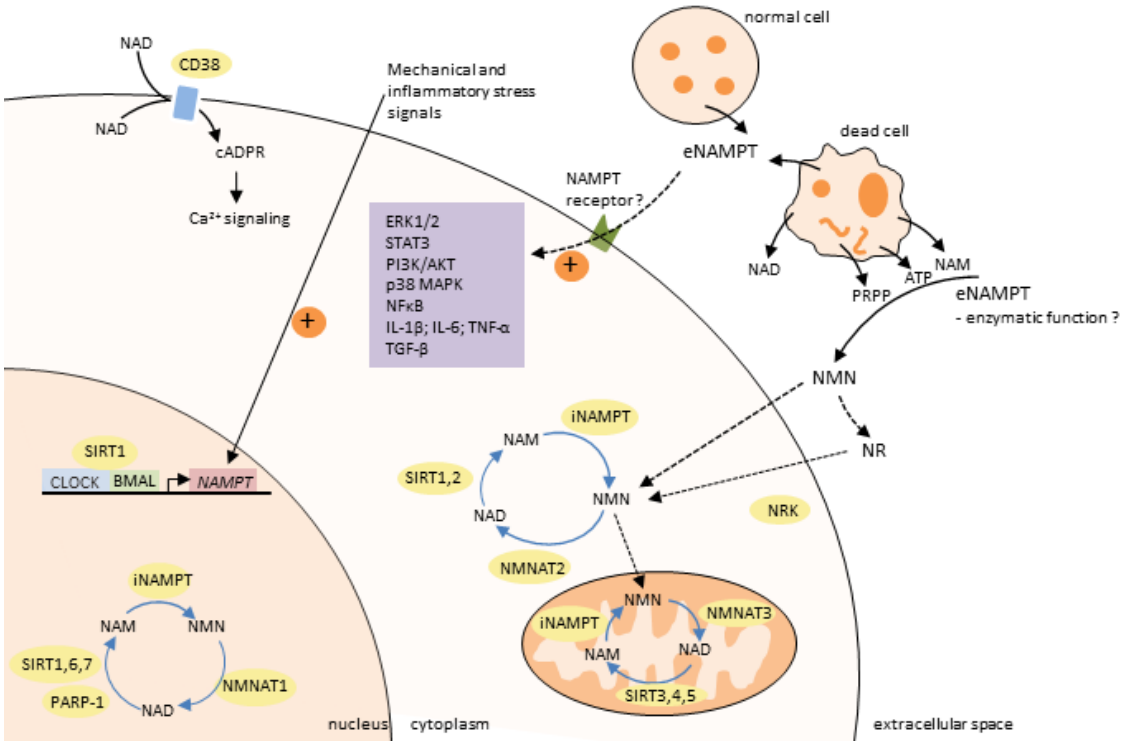

Figure 2. Overview on physiological actions of NAMPT. Intracellular NAMPT (iNAMPT) has been found in the cytoplasm, nucleus and mitochondria. NAMPT exerts its effects by maintaining intracellular NAD levels and recycling nicotinamide produced by the action of NAD-dependent enzymes like sirtuins (SIRTs)-1,-6,-7 and poly-(ADP-ribose) polymerase (PARP)-1 in the nucleus, SIRT-1,-2 in the cytoplasm and SIRT-3,-4,-5 in the mitochondria. The ectoenzyme CD38 produces cADPR (cyclic ADP-ribose) and regulating intracellular Ca^{2+} signaling. NAMPT expression is induced by the circadian regulators CLOCK and BMAL in complex with SIRT1. Other stimulators of NAMPT expression include mechanical stress and pro-inflammatory cytokines (TNFα, IL-1β, IFNγ). NAMPT is secreted from different cell types and most likely released from dying cells together with ATP and 5'-phosphoribosyl-1-pyrophosphate (PRPP). Therefore, a potential extracellular production of nicotinamide mononucleotide (NMN) would take place that subsequently may enter the cell, possibly after being converted into nicotinamide riboside (NR). Apart from enzymatic activity, extracellular NAMPT (eNAMPT) was shown to act as a pro- or anti-inflammatory cytokine on multiple signaling pathways, such as extracellular signal regulated kinase (ERK)1/2, IL-6/Signal transducer and activator of transcription (STAT)3, phosphoinositide-3-kinase (PI3K)/AKT, p38 mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), influencing the expression of several cytokines (TNF-α, IL-1β, TGFβ). An eNAMPT receptor is not yet identified.

2.2 NAMPT in Obesity and non-alcoholic fatty liver disease (NAFLD)

NAMPT and white adipose tissue in obesity

White adipose tissue (WAT) is a key regulator in the development of obesity and obesity-related disorders by operating as a fat storage organ and releasing fatty acids and adipokines [83] into the circulation. Thus, WAT acts as a functional endocrine unit [84]. Ever since NAMPT was described as adipocytokine with insulin-mimetic effects [4], the discussion about the role of NAMPT in obesity and obesity-related disorders has been continued. Different adipocyte models were shown to release NAMPT into the supernatant via a non-

classical pathway [17, 69, 85], identifying adipose tissue as a source of circulating NAMPT. A meta-analysis including human studies on the association of NAMPT with obesity parameters found that circulating NAMPT levels were generally increased in subjects with obesity [86]. Human studies on the association of NAMPT with parameters of obesity published during the last five years are summarised in **table 2**.

Table 2: Obesity and NAFLD.

NAMPT in human studies	NAMPT correlates with
↑ Serum[a] and liver[b] in obese women (n=95) compared to normal weight women (n=38) ↑ Serum[a] and liver[b] in obese women with NAFLD compared to obese women without NAFLD	[a]IL-6 (r=0.496; p<0.001) [a]CRP levels (r=0.241; p=0.049) [b]resistin (r=0.436, p=0.018) [b]TNFα (r=0.328, p=0.028) [148]
± Serum in subjects with NAFLD (n=114) compared to healthy non-diabetic controls (n=60)	TNFα (r = -0.236, p = 0.011) [149]
↓ Serum after 6 weeks weight reduction (n=35)	No correlation [150]
± Serum in obese healthy women (n=43) compared to normal weight women (n=43)	carbohydrate intake (p=0.018, r=0.257) [151]
↑ Serum in obese children (n=44) compared to normal weight children (n=50)	BMI(r=0.247, p=0.029) (boys) IL-6 (r=0.427, p=0.013) (boys) [152]
± Serum in higher-adiposity children and adolescents (n=67) (BMI=23.5 ±2.8 kg/m) compared to lower-adiposity group (BMI BMI=17.6±2.2 kg/m) (n=68)	abdominal fat, visceral fat, subcutaneous abdominal fat, plasma triglyceride level and HOMA-IR (lower-adiposity group) IL-6 (higher-adiposity group) [153]
↑ Serum in obese children and adolescents (n=30) compared to normal weight children and adolescents (n=60)	No correlation [154]
↑ Serum in subjects with NAFLD (n=30) compared to controls without NAFLD (n=27)	No correlation [155]

↑ Serum in morbidly obese women (n=93) compared to lean women (n=40)[a] ↓ Serum 12 months after bariatric surgery[b] ↑ Subcutaneous and visceral adipose tissue[c] in morbidly obese women compared to lean women	[a]IL-6 (r = 0.474, P < 0·001) [a]CRP (r = 0.239, P = 0.042) [b]TNFα (r = 0.434, P = 0.009) [b]IL-6 (r = 0.458, P = 0·006) [c]TNFα (r = 0.314, P = 0.075) [c]IL-6 (6 r = 0.747, P < 0.001) [156]
↑ Serum in exogenous obese pubertal adolescents (n=40) compared to healthy adolescents (n=20)	body weight (r=0.4, p=0.001), BMI (r=0.5, p=0.001), waist-to-hip ratio (r=0.5, =0.001) Serum triglycerides (r=0.3, p<0.05), LDL-C levels (r=0.3, p<0.05) Insulin (r=0.45, p=0.001), C-peptide (r=0.4, p<0.001), HOMA-IR (=0.4, p=0.01), glucose/insulin ratio (-r=0.4, p=0.002) [157]
↑ Serum[a] and human peripheral blood cells[b] in obese subjects compared to lean subjects	[a]BMI (r=0.31; P=0.044), WHR (r=0.47; P=0.012)a [b]BMI (r=0.50; P=0.009), body fat percentage (r=0.48; P=0.014), waist-to-hip ratio (r=0.43; P=0.032)b [a]ALT (r=0.41, P= 0.013), AST(r= 0.41, P= 0.014), γ-GT (r=0.41, P= 0.014)a [b]HIF1-α mRNA (r=0.45; P=0.030) [158]
↑ Serum in obese children (n=14) compared to lean children (n=15)	leucocyte counts (r=0.46, p<0.001) [27]
↑ Serum in adult obese subjects (n=46) compared to control group (n=44)	BMI (P<0.05), waist circumference, (P<0.001), hip circumference (P<0.001), HOMA (P<0.05) [159]
↑ Serum in obese children and adolescents (n=88) compared to lean children and adolescents (n=22)	waist circumference (r = 0.36, P = 0.036), BMI% (r = 0.38, P = 0.025) (boys) whole body insulin sensitivity index (r = -0.36, P = 0.036) (boys) IL-6 (r = 0.38, P = 0.024), thiobarbituric acid reactive substances (r = 0.52, P = 0.001) (boys) [160]
↑ Serum in prepubertal obese children compared (n=100) to control children (n=42)	Resistin, IL-6 [161]
↓ Serum and liver in subjects with NAFLD (n=58) compared to healthy controls (n=27)	No correlation [100]

↓ Visceral adipose tissue in subjects with NAFLD (n=77) compared to subjects without NAFLD (n=38)	No correlation [162]
↑ Liver in subjects with fibrosis (n=33) compared to non-fibrosis subjects with NAFLD (n=7)	fibrosis stage (r = 0.52, p = 0.03) [163]
± Serum in obese women compared to lean women	No correlation [164]
↑ Serum in obese subjects (n=68) compared to healthy non-obese subjects (n=30)	age (r = -0.26, p = 0.034), waist-to-hip ratio (r = -0.28, p = 0.031) HbA(1c) (r = -0.36, p = 0.0037) [165]
↓ Subcutaneous tissue in obese subjects (n=31) compared to control subjects (n=31)	No correlation [166]

↑up and ↓down regulation of NAMPT; ± no change

Several metabolic factors found to be increased in obese subjects were also shown to influence NAMPT expression and/or release. Both glucose and oxidized low density lipoprotein was shown to stimulate NAMPT expression and release in human adipocytes via the PI3-kinase/AKT pathway [87–89]. In addition, glucose administration in human subjects led to increased eNAMPT levels [27]. Further *in vitro* studies showed that *NAMPT* mRNA expression increased during adipogenesis [90] and was stimulated by insulin resistance-inducing factors like IL-6, dexamethasone, growth hormone, TNFα and isoproterenol [49, 91]. The macrophage population in obese human visceral WAT was identified as another source of eNAMPT [92]. On the other hand, eNAMPT was found to induce an up-regulation of pro-inflamatory cytokines like monocyte chemoattractant protein (MCP)-1, an adipocyte-secreted protein which might play a crucial role in metabolic and vascular disease, in WAT [93]. The pro-inflammatory effects of eNAMPT in adipocytes might be mediated through the IL-6-TRAF6-NF-ϰB pathway[94]. NAMPT was also described to stimulate tumor necrosis factor alpha (TNFα) and IL-6 expression [33]. Furthermore, eNAMPT increased lipoprotein lipase and peroxisome proliferator-activated receptor gamma (PPARγ) as well as fatty acid synthase in preadipocytes and differentiated adipocytes, respectively, suggesting NAMPT as a regulator of lipid metabolism [95] as well as indicating a role of NAMPT in the development of obesity and related disorders.

NAMPT and liver in obesity

NAMPT seems to play a role in the development of non-alcoholic fatty liver disease (NAFLD), which is the most common liver disorder in western countries. NAFLD covers a wide range of pathophysiological conditions from simple steatosis (hepatic lipid accumulation) to inflammation (non-alcoholic steatohepatitis), which frequently leads to fibrosis and cirrhosis accompanied with a higher risk for hepatocellular carcinoma and eventually the need for liver transplantation [96]. We could identify hepatocytes as a source of eNAMPT [70]. It was shown that NAMPT plasma concentrations correlated with portal inflammation in subjects with NAFLD [97]. Several, partly conflicting, studies about the association of NAMPT with severity of NAFLD have been published during the following years and are summarized in **table 2**, with NAMPT up and down regulation both being observed in animal models or human subjects with NAFLD. In the liver, NAMPT is regulated by FoxO transcription factors, that are part of the insulin signaling network and implicated in the regulation of adipogenesis and gluconeogenesis [98]. Liver specific (FoxO)-1, 3 and 4 knockout mice were shown to have increased hepatic triglyceride and reduced NAMPT levels. Overexpression of NAMPT significantly reduced hepatic triglycerides *in vivo* [43]. Another regulator of NAMPT is miRNA-34a that was shown to be elevated in obese subjects and significantly reduced NAD levels and SIRT1 activity in the liver by directly targeting *NAMPT* mRNA expression. In diet-induced obese mice inhibition of miR-34 restored Nampt and NAD levels and improved steatosis, inflammation, and glucose intolerance [99]. Apoptosis was shown to be improved in stress-exposed hepatocytes if NAMPT was over expressed. Furthermore, pharmacological activation of peroxisome proliferator-activated receptors (PPAR) α, which is up regulated in patients with NAFLD and is a major regulator of lipid metabolism in the liver, led to attenuated Nampt levels in rat hepatocytes [100]. Troxerutin, a trihydroxyethylated derivative of the natural bioflavonoid rutin, prevented obesity, liver steatosis and injury in high-fat diet treated mice by enhancing Nampt protein and NAD levels, decreasing Parp protein levels and restoring Sirt1 activity [101]. Supplementation of leucine, an essential amino acid, improved Nampt and Sirt1 protein levels as well as NAD levels in high-fat diet fed mice. This effect was underlined by a decrease of the acetylation status of the Sirt1 targets PPARγ coactivator 1-alpha (PGC-1α) and FoxO1 suggesting improved mitochondrial biogenesis as well as insulin sensitivity and glucose metabolism, respectively [102]. In contrast, a pro-inflammatory action of NAMPT was found in HepG2 cells. When treated with palmitate a time-and dose-dependent increase of NAMPT mRNA and protein expression as well as mRNA expression of *IL-6* and *TNFa* was found while a down

regulation of NAMPT counteracted the inflammatory response. By inhibition of NF-κB, NAMPT protein levels were normalized after stimulation with palmitate. This indicates that NAMPT might play a role in palmitate-induced inflammation in hepatocytes through the NF-κB pathway [103]. Another *in vitro* study using HepG2 cells showed that eNAMPT activated gluconeogenesis via activation of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, which did not depend on SIRT1 activation [64].

Taken together, NAMPT is able to modulate important processes in the pathogenesis of NAFLD by regulating oxidative stress (or mitochondrial biogenesis), apoptosis, lipid and glucose metabolism, inflammation and insulin resistance.

2.3 NAMPT and type 2 diabetes (T2D)

The increasing prevalence of obesity is contributing substantially to the ongoing epidemic of T2D [104, 105]. It is well demonstrated that increased abdominal adiposity is associated with low-grade inflammation, abnormal hormone secretion and various metabolic disturbances that contribute to the genesis of insulin resistance [104, 105]. Recently, there has been a growing interest on the role of different adipokines in the pathogenesis of metabolic complications related to obesity [106, 107]. In particular, in the large spectrum of adipokines, NAMPT represents one of the most promising and interesting molecules that seems directly implicated in the regulation of glucose-stimulated insulin secretion (GSIS) in pancreatic β cells [17].

NAMPT and T2D in animal models

While some studies explained the effects of Nampt on glucose metabolism by the controversial finding of insulin-mimetic actions of Nampt [108], others focused on Nampt as NAD biosynthetic enzyme. Circulating levels of the Nampt product NMN and consequently NAD levels in beta cells/islets were significantly lower in old beta cell-specific Sirt1-overexpressing (BESTO) mice compared to young BESTO mice. NMN administration restored the positive effect of Sirt1 on glucose tolerance and glucose-stimulated insulin secretion (GSIS), at least in the aged BESTO female mice [109]. A defect in NAD biosynthesis and GSIS was found in islets of $Nampt^{+/-}$ mice and an impaired glucose tolerance in $Nampt^{+/-}$ female mice. These alterations in glucose metabolism were ameliorated after the administration of NMN, one of the products of Nampt enzymatic reaction and a key NAD intermediate. This result suggested that the observed defects in GSIS were due to a reduction of Nampt -mediated NAD biosynthesis. To confirm these

data, FK866, a potent NAMPT inhibitor, significantly inhibited NAD biosynthesis and GSIS in wild-type islets, while administration of NMN restored normal NAD biosynthesis and GSIS in FK866-treated wild-type islets. Taken together, these findings strongly suggest that Nampt is able to control the regulation of insulin secretion by Nampt-mediated NAD biosynthesis in pancreatic beta cells [17]. Confirming this hypothesis, we demonstrated that NAMPT and NMN were able to induce a 2.0- and 1.8-fold increase of insulin secretion compared to glucose alone in human islets after 1 h incubation with high glucose concentration [110]. In a high-fat diet mouse model, a direct effect of Nampt not only in glucose metabolism but also in the pathogenesis of T2D was found. Nampt-mediated NAD biosynthesis was shown to be impaired in high-fat diet mice with diabetes compared to regular chow-fed control healthy mice, while the administration of NMN ameliorated glucose intolerance by restoring NAD levels. In addition, NMN augmented hepatic insulin sensitivity and other biological pathways related to oxidative stress, inflammation and lipid metabolism [111]. Fructose-rich diet (FRD)-fed mice showed increased levels of Nampt in brown and white adipose tissue but significantly lower levels of circulating Nampt. Administration of NMN abolished the suppressive effects of FRD on insulin secretion. NMN also showed protective effects against pro-inflammatory cytokine-mediated islet dysfunction. Insulin secretion in islets cultured with pro-inflammatory cytokines was restored by NMN. The anti-inflammatory effects of NMN were partially blocked by inhibition of Sirt1 [112]. Another NAD intermediate, NR, was also found to be beneficial by ameliorating NAD levels. HFD-induced body weight gain in mice was significantly attenuated by NR which was due to enhanced energy expenditure [113]. In contrast to NMN there are three clinical trials registered for NR (ClinicalTrials.gov) such as measuring serum NR and metabolites of NR (NCT02300740), analysing NR and its metabolites in urine and blood (NCT02191462) or investigating the insulin sensitivity and substrate metabolism in obese men after a 3 months treatment with NR (NCT02303483).

NAMPT and T2D in humans

Recently there has been a growing body of evidence showing a possible association between NAMPT levels and T2D in obese and non-obese patients [114–116]. Several studies reported that subjects with T2D presented significantly higher levels of NAMPT compared to healthy controls, independently of body mass index or adipose tissue. In addition, all these studies confirmed with multiple logistic regression analysis that NAMPT could be considered as an independent factor for T2D, even after adjustment for other risk factors [115, 117, 118]. In order to define the relationship between NAMPT and beta cell

impairment, levels of NAMPT were compared in three different groups: adult patients with type 1 diabetes, patients with T2D and healthy controls. In this study the authors reported that circulating NAMPT was significantly increased in type 1 diabetics as compared to T2D and non-diabetic subjects, underlining a possible inverse relationship between deterioration of beta cell function and NAMPT levels [119]. Although these data are very interesting, some limitations of this study need to be considered. In particular, the cross-sectional design permits to identify only a correlation between variables, but not any direct cause and effect. Therefore, it would be interesting to measure changes of plasma NAMPT levels in obese subjects during different stages of diabetes development to further clarify the role of this adipokine in the pathogenesis of T2D.

NAMPT and T2D comorbidities

More recently it was found that NAMPT not only is involved in the pathogenesis of beta cell dysfunction and diabetes but it also seems to play an important role in diabetes complications [106, 120]. In particular, patients with T2D were shown to present increased levels of NAMPT compared with controls, but more interestingly a significant correlation between NAMPT and signs of endothelial dysfunction have been proposed [120]. In addition, NAMPT levels were also increased in other complications such as diabetic nephropathy or lipid impairments [116]. A possible association between different NAMPT polymorphisms and T2D or T2D-related complications [29, 121] was reported. A higher frequency of NAMPT -948G/G genotype could be observed in T2D patients compared with controls. Furthermore, the frequency of this genotype was significantly higher in T2D patients with cardiovascular disease than in those without [121]. Human studies on the association of NAMPT with diabetes published during the last five years are summarised in **table 3**. All these evidences in *in vitro* and *in vivo* models demonstrated the powerful role of NAMPT in the pathogenesis of diabetes and in the development of diabetes related complications. More interestingly, *in vitro* studies reported an important role of NMN in the amelioration of beta cell function and in cellular homeostasis, glucose metabolism and stress responses.

Table 3: NAMPT and T2D.

NAMPT	NAMPT correlates with
↑ NAMPT in obese patients with T2D (n=19) ***vs*** non obese (n=37) with T2D ***vs*** controls (n=19)	BMI (r=0.434; p<0.001), WHR (r=0.280; p=0.015), FPG (r=0.283; p=0.014), HOMA-IR (r=0.357; p=0.002), TG (r=0.282; p=0.014), TC (r=0.278; p=0.016), IL-6 (r=0.431; p<0.0001), vaspin (r=0.505; p<0.0001) [117]
↑ NAMPT in obese patients with T2D (n=61) ***vs*** controls (n=59)	Multiple linear regression for NAMPT and T2D: Model adjusted for age, gender (OR=3.247; 95%CI 1.426–7.394; p=0.005) Model adjusted for age, gender, BMI, WHR (OR=2.320; 95%CI 1.000–4.695; p=0.043) Model adjusted for age, gender, BMI, WHR, SBP, DBP (OR=2.895; 95%CI 1.113–7.529; p=0.029) Model adjusted for age, gender, BMI, WHR, SBP, DBP, lipid profile, smoking status (OR=5.534; 95%CI 1.605–19.079 p=0.007) [118]
↑ NAMPT in type 1 diabetic patients with long-standing disease (n=58) and in T2D patients (n=64) ***vs*** nondiabetic subjects(n=118)	In **nondiabetic subjects** with Acute insulin response to glucose (r=0.27, p=0.002) 30-min OGTT insulin (r=0.24, p=0.005) Fasting insulin (r=0.20, p=0.042) Insulin sensitivity (r=0.19, p=0.043)
↑ NAMPT in patients with long-standing disease type 1 diabetic (n=58) ***vs*** patients T2D (n=64)	In subjects with **type 2 diabetes** HbA1C (β= 0.457, p=0.0001) In subjects with **type 1 diabetes** Age (but not sex, BMI, or HbA1C) (β=0.344, p= 0.010) no correlation between serum visfatin concentration and HbA1C in type 1 diabetic subjects (r=0.17, p=0.20) [119]
↑ NAMPT in patients with T2D (n=85) and minor (<500 mg/day) or severe proteinuria (n=45) (≥500 mg/day) ***vs*** with age- and sex-matched controls (n=38) ↑ NAMPT in patients with T2D and severe proteinuria (n=45) (≥500 mg/day) ***vs*** patients with T2D and minor proteinuria (n=40) (<500 mg/day)	In subjects with **type 2 diabetes** Proteinuria (r= 0.46, p<0.0001) Flow mediated dilatation (r=-0.47; p<0.001) [120]

↑ NAMPT in patients with T2D an CAD (n=195) compared to subject with T2D without CAD (n=56)	prevalence of CAD in type 2 diabetic patients by tertiles of plasma NAMPT was 57% (T1), 67% (T2) and 75% (T3) Positive association between highest tertile of plasma NAMPT and CAD:
± NAMPT in non-diabetic subjects with or without CAD (n=98)	OR 2.09, 95% CI 1.11 – 4.01, p = 0.02 (T3 vs T1) OR 1.62, 95% CI 0.83 – 3.19, p = 0.15 (T3 vs T2) OR 1.29, 95% CI 0.68 – 2.44, p = 0.42 (T2 vs T1) T-allele of rs9770242 NAMPT gene polymorphism was associated with CAD in Brazilian cohort (OR 1.46, 95% CI 1.06 - 2.01, p = 0.02) while no association was observed in North-American cohort [29]
↑ NAMPT in patients T2D (n=44) without CVD and (n=46) with CVD ***vs*** healthy control subjects (n=60)	NAMPT -948G/G genotypes and G alleles significantly associated with T2DM (OR 2.94, 95% CI 1.06 – 8.17, p = 0.039) and CVD in diabetic patients (OR 3,56, 95% CI 1.10 – 11,51, p = 0.034) [121]
↑ NAMPT in T2D patients with CVD and with NAMPT -948G/T polymorphism *vs* T2D with CVD and without NAMPT -948G/T polymorphism	

↑up and ↓down regulation of NAMPT; ± no change

BMI: body mass index, WHR: waist to hip ratio, FPG: fasting plasma glucose, HOMA-IR: homeostatic model assessment – insulin resistance, TG: triglycerides, TC: total cholesterol, OR: odds ration, CAD: coronary artery disease, CI: confidence interval, SBP: systolic blood pressure, DBP: diastolic blood pressure, OGTT: oral glucose tolerance test, HbA1C: glycated hemoglobin, CVD: coronary vascular disease

2.4 NAMPT in senescence, aging and cancer

NAMPT in aging

Sirtuins have been comprehensively investigated as mediators of longevity [122]. NAMPT as a regulator of sirtuin function has been shown to delay cellular senescence by increasing oxidative stress resistance in human vascular smooth muscle cells [123]. In contrast, extracellular NAMPT induced telomere damage and premature senescence in human endothelial cells by activation of NADPH oxidase [124].Reduced levels of Nampt and NAD have been shown in peripheral tissue of aging mice, such as pancreas, WAT and skeletal muscle. In this study evidence was provided that administering of the Nampt enzyme product, NMN, could be an effective intervention to treat the pathophysiology of age-induced T2D [111]. Interestingly, aging also reduced Nampt-mediated NAD

biosynthesis in the brain, particularly in the hippocampus, affecting the function of neuronal stem neural stem/progenitor cells (NSPC). Aging is one of the strongest negative regulators of adult NSPC proliferation [125]. NSPC possess the ability to proliferate and differentiate into major cells of the brain, such as produce neurons, oligodendrocytes or astrocytes. Thus, they contribute to cognitive function and can be reactivated in the aged brain [125]. In this study, Nampt was shown to be involved in the molecular mechanism that leads to the decline of NSPC during aging. The authors raised the idea of a long-term NMN administration that may counteract age-related declines in NSPC functionality [126].

A study on young and old Wistar rats demonstrated that Nampt levels were decreased in the aged group. Interestingly, the age-associated decrease in Nampt and NAD levels were reversed with regular exercise, leading to increased specific activity of Sirt1 [52]. A recent clinical study on a large population of elder people investigated the relationships between circulating NAMPT levels, nutritional status, and insulin resistance. They found that plasma NAMPT levels decline with age and are related to nutritional status, especially visceral obesity, and inflammation [127].

There are several theories trying to explain the decline of NAMPT and NAD in aging. As discussed above, NAMPT is a major output of the circadian transcription factors BMAL and CLOCK [40]. One hypothesis is that a decline in central and peripheral circadian function during aging would result in a deficit in NAMPT and NAD production. Aging is accompanied by a state of chronic, low-grade inflammation, which is a major contributor to the development of many age-related chronic disorders. Interestingly, it has been shown that TNF-α, one of the major inflammatory cytokines, and oxidative stress significantly reduce NAMPT and NAD levels in primary hepatocytes [111]. TNF-α also suppresses CLOCK/BMAL-mediated clock gene functions in mice [128]. This in turn may be another reason why NAMPT-mediated NAD biosynthesis is decreased during aging. As mentioned above, chronic inflammation, oxidative stress and DNA damage are crucial players associated with aging. The activation of NAD-dependent PARPs is induced immediately after DNA damage to facilitate repair and maintenance of genomic integrity. Thus, acute DNA damage can induce a sudden depletion of NAD due to PARP activation. During aging, damaged DNA accumulates in the nucleus, causing PARP activation and might be another possible explanation for age-induced NAD reduction. Most recently, several studies on whole-body Sirt6 transgenic mice or brain-specific Sirt1 overexpression demonstrated the importance of sirtuins in aging [129, 130].

NAMPT in cancer

NAMPT-mediated NAD production is essential for cellular metabolism, energy production, and DNA repair - processes that undergo crucial changes during malignant transformation. Cancer cells possess a high glucose uptake and an increased rate of aerobic glycolysis. These metabolic alterations require increased amounts of the redox co-factor NAD. There is a strong body of evidence that cancer cells have a higher NAD turnover than normal cells due to their increased energy demand used for cell proliferation and DNA repair. As mentioned above, NAD functions in many critical cellular events that are necessary for cancer cell growth, including transcriptional regulation, cell cycle progression, apoptosis, DNA repair, chromatin dynamics regulation and telomerase activity [59]. As NAD is rapidly consumed in cancer cells and converted to nicotinamide, NAMPT is essential for the replenishment of the intracellular NAD pool. Therefore, the development of many cancers is associated with increased NAMPT expression including colorectal, ovarian, breast, gastric, prostrate, well-differentiated thyroid, and endometrial carcinomas, myeloma, melanoma and astrocytomas [131]. Recently, we could show that NAMPT is differentially expressed in hepatocarcinoma cell lines as compared to non-cancerous human hepatocytes. Resveratrol treatment of hepatocarcinoma cell lines led to decreased NAMPT activity and cell death, while in non-cancerous human hepatocytes NAMPT activity and NAD levels were increased upon incubation with resveratrol [71]. In a meta-analysis of genome-wide expression data to identify NAMPT-influenced genes, a reduced NAMPT expression was found to strongly dysregulate cancer biology signaling pathways [132]. Cancer cells were demonstrated to be more susceptible to NAMPT inhibition than normal cells [133, 134].

Clinical studies also demonstrated that serum or blood levels of NAMPT were found to be increased in cancer patients with a positive correlation between either tissue or circulating levels and stage progression [34, 135–137]. However, the molecular mechanism of eNAMPT on carcinogenesis is far from clear. A recent study demonstrated that eNAMPT affected redox adaptive responses and promoted tumor proliferation in human malignant melanoma cells [138]. Furthermore, cell lines overexpressing NAMPT were significantly more resistant to chemotherapeutic agents than control cells [139]. In contrast, stable NAMPT knock-down cells were shown to be more sensitive to such treatment than controls [16]. NAMPT was shown to activate the MAPK/ERK pathway and to stimulate the vascular endothelial growth factor (VEGF) promoting angiogenesis, a crucial process during tumor growth and expansion. Targeting NAMPT activity represents a novel therapeutic strategy for human cancers. For example, the specific NAMPT inhibitor

FK866 has been evaluated in a broad variety of tumors, including solid tumors and leukemia [140–142] *in vitro* and nude mouse xenografts [25, 140, 143–145], in which FK866 was able to reduce or attenuate tumor growth. NAMPT inhibition attenuated glycolysis in conjunction with the reduction of NAD levels and led to a blockade of the pentose phosphate pathway, serine biosynthesis, and the tricarboxylic acid cycle [145]. In a recent study, we could demonstrate that FK866 induced delayed energy stress in hepatocarcinoma cells that triggered the activation of AMPK and down regulated mTOR signaling which was associated with increased cancer cell death. Non-cancerours human hepatocytes were less sensitive to FK866 [146]. Clinical trials applying NAMPT inhibitors as monotherapy (summarised in **table 4**) have so far been less promising. One possible explanation could be the action of CD38 or CD73 reversing cell death induced by NAMPT inhibition through the supply of ectocellular NAD precursors [147]. However, combining FK866 or other NAMPT inhibitors with antineoplastic agents, chemotherapy or radiotherapy might enhance their therapeutic efficacy.

Table 4: Clinical trials inhibition of NAMPT

Inhibitor		
CHS 828:	**CHS 828 in Treating Patients With Solid Tumors (Phase 1)**	
	ClinicalTrials Identifier:	NCT00003979
	Condition:	Unspecified Adult Solid Tumor, Protocol Specific
	Status:	This study has been withdrawn prior to enrollment.
	Safety and efficacy of NAD depleting cancer drugs: results of a phase I clinical trial of CHS 828 and overview of published data. (Phase 1) [167]	
	Condition:	advanced solid tumours
	Status:	This study has been completed.
GMX1777:	**A Phase I/II Study of GMX1777 in Combination With Temozolomide for the Treatment of Metastatic Melanoma (Phase 1; Phase 2)**	
	ClinicalTrials Identifier:	NCT00724841
	Condition:	Metastatic Melanoma
	Status:	This study has been terminated. (Study terminated prematurely due to financial contraints.)

	Safety and Efficacy of GMX1777 in the Treatment of Refractory Solid Tumors or Lymphomas (Phase 1)	
	ClinicalTrials Identifier:	NCT00457574
	Condition:	Solid Tumors and Lymphomas
	Status:	This study has been withdrawn prior to enrollment. (Study terminated prematurely due to financial constraints.)
APO866 (FK866)	**A Study of APO866 for the Treatment of Cutaneous T-cell Lymphoma (Phase 2)**	
	ClinicalTrials Identifier:	NCT00431912
	Condition:	Cutaneous T-cell Lymphoma
	Status:	This study has been completed. (No study results posted)
	A Phase I/II Study to Assess the Safety and Tolerability of APO866 for the Treatment of Refractory B-CLL (Phase 1; Phase 2)	
	ClinicalTrials Identifier:	NCT00435084
	Condition:	B-cell Chronic Lymphocytic Leukemia
	Status:	This study has been completed. (No study results posted)
	A Study to Assess APO866 for the Treatment of Advanced Melanoma (Phase 2)	
	ClinicalTrials Identifier:	NCT00432107
	Condition:	Melanoma
	Status:	This study has been completed. (No study results posted)
	The pharmacokinetics, toxicities, and biologic effects of FK866, a nicotinamide adenine dinucleotide biosynthesis inhibitor[168]	
	Condition:	advanced solid tumor malignancies refractory to standard therapies
	Status:	This study has been completed.

2.5 Conclusions

- By regulating intracellular NAD concentrations NAMPT is involved in regulating cellular energy metabolism.
- NAD concentrations are decreased in various metabolic disorders and during aging.
- Application of NAD precursors seems to be successful in augmenting NAD levels under pathophysiological conditions and aging.
- NAMPT regulates glucose-stimulated insulin secretion (GSIS) in pancreatic beta cells.
- NAMPT regulates central pathogenic mechanisms during the progression of NAFLD.
- NAMPT plays a crucial role in cancer cell metabolism and is often overexpressed in tumor tissues.

2.6 Open questions

- Are there other functions besides the regulation of NAD concentrations for intracellular NAMPT?
- How is NAMPT secretion regulated and what is the role of circulating NAMPT/eNAMPT?
- Is eNAMPT enzymatically active under pathophysiological conditions?
- Does NAMPT expression and function depend on disease progression and severity in metabolic disorders?
- Why does NAMPT inhibition lead to cell death and tumor remission *in vitro* and in animal models, but not in clinical trials?

Detailed author contributions can be found at the end of this work.

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CHAPTER 3

FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signaling in hepatocarcinoma cells

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Background: Nicotinamide phosphoribosyltransferase (NAMPT) is the key enzyme of the NAD salvage pathway starting from nicotinamide. Cancer cells have an increased demand for NAD due to their high proliferation and DNA repair rate. Consequently, NAMPT is considered as a putative target for anti-cancer therapies. There is evidence that AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) become dysregulated during the development of hepatocellular carcinoma (HCC). Here, we investigated the effects of NAMPT inhibition by its specific inhibitor FK866 on the viability of hepatocarcinoma cells and analysed the effects of FK866 on the nutrient sensor AMPK and mTOR complex1 (mTORC1) signaling.

Results: FK866 markedly decreased NAMPT activity and NAD content in hepatocarcinoma cells (Huh7 cells, Hep3B cells) and led to delayed ATP reduction which was associated with increased cell death. These effects could be abrogated by administration of nicotinamide mononucleotide (NMN), the enzyme product of NAMPT. Our results demonstrated a dysregulation of the AMPK/mTOR pathway in hepatocarcinoma cells compared to non-cancerous hepatocytes with a higher expression of mTOR and a lower AMPK activation in hepatocarcinoma cells. We found that NAMPT inhibition by FK866 significantly activated AMPKα and inhibited the activation of mTOR and its downstream targets p70S6 kinase and 4E-BP1 in hepatocarcinoma cells. Non-cancerous hepatocytes were less sensitive to FK866 and did not show changes in AMPK/mTOR signaling after FK866 treatment.

Conclusion: Taken together, these findings reveal an important role of the NAMPT-mediated NAD salvage pathway in the energy homeostasis of hepatocarcinoma cells and suggest NAMPT inhibition as a potential treatment option for HCC.

3.1 Introduction

The co-factor nicotinamide adenine dinucleotide (NAD) plays a crucial role in multiple cellular processes and is substrate for a variety of enzymes and regulatory proteins [1]. In humans a main portion of NAD is generated via the nicotinamide (NAM) salvage pathway, in which nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step in the biosynthesis of NAD yielding nicotinamide mononucleotide (NMN) [2;3]. As NAD is rapidly consumed in cells (1h) and converted to NAM [4], NAMPT is essential for the replenishment of the intracellular NAD pool. The development of many cancers is associated with increased NAMPT expression [5]. Cancer cells have a high rate of NAD turnover due to their increased energy demand and a high activity of NAD-dependent enzymes, such as poly (ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs) and sirtuins, required for DNA repair, genome stability and proliferation [1;5]. Therefore, cancer cells are more susceptible to NAMPT inhibition than normal cells [6;7]. In previous studies, we found that NAMPT is released from hepatocytes [8] as well as differentially expressed and more enzymatically active in hepatocarcinoma cells compared to non-cancerous human hepatocytes [9]. Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths [10]. The only available proven systemic therapy for HCC is the multi-targeting kinase inhibitor sorafenib [11]. An effective second-line agent for patients with sorafenib failure or intolerance has yet to be identified. This has led to an intensive search for molecular pathways and novel compounds for the treatment and prevention of HCC. Targeting NAMPT activity and intracellular NAD content represents a novel therapeutic concept for HCC. The specific NAMPT inhibitor FK866 is a competitive inhibitor that was selected by an anticancer screening system differentiating acute cytotoxicity from growth inhibition [12;13]. FK866 has been evaluated in a broad variety of tumors, including solid tumors and leukemia [14-16] *in vitro* and in nude mouse xenografts [17-19], where FK866 was able to reduce or attenuate tumor growth.

In HCC tissue, AMP-activated protein kinase (AMPK), a major regulator of cellular energy homeostasis that coordinates multiple metabolic pathways, has been shown to be dysregulated compared to normal tissue [20;21]. AMPK activity opposes tumor development and negatively regulates the Warburg effect (aerobic glycolysis) leading to suppression of tumor growth *in vivo* [20-22]. AMPK translates changes in glucose availability and fluctuation of energy to mammalian target of rapamycin (mTOR) and thereby acts as a master energy sensor to modulate cellular activities in response to energy stress [23;24]. mTOR, a serine/threonine protein kinase, has been observed to be increased in multiple human cancers, including HCC, where it is associated with less differentiated

tumors, earlier tumor recurrence, and worse survival outcomes [25;26]. Inhibition of mTOR has proven efficacious in clinical trials [26;27]. Recently, there is great scientific interest in finding molecular pathways and novel compounds that target AMPK/mTOR signaling as a new treatment option for HCC.

Little is known about the interaction of NAMPT and AMPK/mTOR signaling during the development of HCC. In this study, we investigated the effects of the NAMPT inhibitor FK866 on hepatocarcinoma cells and non-cancerous human hepatocytes. We asked whether or not FK866-induced energy stress might activate AMPK and modify the mTOR signaling pathway and whether the observed effects could be rescued by the NAMPT enzyme product NMN.

3.2 Material and Methods

Material

Cell culture media, supplements and antibiotics were obtained from PAA (Cölbe, Germany) or Invitrogen (Karlsruhe, Germany). FK866, nicotinamide mononucleotide (NMN) and camptothecin were purchased from Sigma-Aldrich (Munich, Germany). Etoposide was purchased from Merck Millipore (Darmstadt, Germany).

Hepatocarcinoma cell lines

Huh7 cells (p53-mutated) and Hep3B cells (p53-deficient) were maintained in DMEM medium with high glucose or MEM medium, repectively. Media were supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100IU penicillin and 100µg/mL streptomycin. All cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Primary human hepatocytes

Tissue samples from patients undergoing liver surgery at the University Medical Center Regensburg were used. Primary human hepatocytes (PHH) were isolated and cultivated as described recently [28]. Briefly, non-neoplastic tissue samples from liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. PHHs were isolated using a modified two-step EGTA/collagenase perfusion procedure and plated on collagen coated dishes. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR

(Human Tissue and Cell Research, Regensburg, Germany), with the informed patient's consent approved by the local ethical committee of the University of Regensburg. All experiments involving human tissues and cells have been carried out in accordance to *The Code of Ethics of the World Medical Association* (Declaration of Helsinki). Cells were seeded in Williams' Medium E containing 2mM glutamine, 10^{-7}mol/L dexamethasone, 100IU penicillin, 100µg/mL streptomycin and 10%FBS. All cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell treatments

FK866 was dissolved in DMSO to create a stock solution of 10mM. NMN was dissolved in the appropriate medium for a stock solution of 100mM. After 16h serum starvation, cells were treated with the indicated concentration of FK866 alone or in combination with NMN [500µM] for 24, 48 and 72h.

Cell viability and apoptosis

Cell viability analysis was conducted using the cell proliferation reagent WST-1 (Roche, Grenzach-Wyhlen, Germany) according to manufacturer´s instructions. To examine the effects of FK866 on cell death, the number of dead cells was measured by FACS analysis at different time points (48h, 72h) using the AnnexinV-FITC Apoptosis Detection Kit (BD Pharmingen™, Franklin Lakes, USA). Adherent and floating cells were analysed according to manufacturer´s protocol. Samples were analysed using a Beckton-Dickinson FACS LSRII. As positive control, apoptosis was induced via camptothecin [2µM] and etoposide [85µM] for 24h. Annexin$^+$ (An^+) and double-stained An^+/propidium iodide (PI^+) cells were considered as dead cells.

ATP measurement

ATP levels were measured with the luminescent-based CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA) according to the manufacturer's protocol.

Western Blot

Protein extraction and Western Blot analysis were performed as described previously [9]. Primary antibodies used for immunoblotting included anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-tubulin, anti-phospho p70S6 kinase (Thr389), anti-p70S6 kinase, anti-phospho-4E-BP1 (Ser65), anti-4E-BP1,

anti-acetylated lysine (Cell Signaling, Beverly, MA, USA) and anti-GAPDH (MerckMillipore, Schwalbach, Germany). Appropriate secondary antibodies were purchased from DAKO (Hamburg, Germany). Immunoblotting for GAPDH or tubulin was performed to verify equivalent amounts of loaded protein. Densitometric analysis was performed using ImageJ 1.41 Software (NIH, USA).

NAMPT enzymatic activity

NAMPT activity was measured by the conversion of ^{14}C- labelled nicotinamide to ^{14}C-NMN using a method previously described [9;29]. Radioactivity of ^{14}C-NMN was quantified in a liquid scintillation counter in counts per minute (cpm) (Wallac 1409 DSA, PerkinElmer). NAMPT activity (cpm) was normalized to total protein concentration as measured by the BCA protein assay.

NAD measurement

Concentrations of NAD from whole-cell extracts were quantified by HPLC analysis using a SUPELCOSIL™ LC-18-T HPLC column (Sigma Aldrich) at a flow rate of 0.8ml/min with 100% buffer A (potassium phosphate buffer pH 6.0) from 0–2min, a linear gradient to 85% Buffer A/15% Buffer B (100% methanol) from 2-5min, 85% Buffer A/15% Buffer B from 5-10min, a linear gradient to 100% Buffer A from 10–12min and 100% Buffer A from 12–15min. NAD was eluted as a sharp peak at 8min and quantitated based on the peak area compared to a standard curve and normalized to total protein concentration as measured by the BCA protein assay.

3.3 Results

FK866-induced NAMPT inhibition significantly decreased NAD levels in human hepatocarcinoma cells which could be ameliorated by NMN administration

We stimulated hepatocarcinoma cells with FK866 [10nM] and found significantly reduced NAMPT activity (-74.9±8.1% in Huh7 cells, -38.1±3.7% in Hep3B cells) (Fig.1A) which caused a sharp decline of NAD levels (Huh7 cells 3.3± 0.3µmol/g protein [con] vs. 0.3±0.2µmol/g protein [10nM FK866]; Hep3B cells 2.2±0.7µmol/g protein [con] vs. 0.2±0.08µmol/g protein [10nM FK866]) (Fig.1B). Co-treatment with NMN restored intracellular NAD levels in all tested cell lines (Fig.1B). To investigate the sensitivity of non-cancerous human hepatocytes towards FK866, we used the same treatment conditions as for hepatocarcinoma cells and found that non-cancerous hepatocytes showed no

significant reduction in NAMPT activity and NAD levels at 10nM FK866 after 48h (Supplement Fig.1A,B).

Emerging evidence suggests that the cellular acetylation state is associated with the energy state of a cell [30]. We could show that FK866-induced NAD depletion led to a decreased activity of NAD-dependent lysine deacetylases as measured by an increased global acetylation of lysine residues (+1.9-fold, $p<0.001$) (Fig.1C). The administration of NMN abrogated the FK866-induced hyperacetylation of lysine residues ($p<0.001$) (Fig.1C).

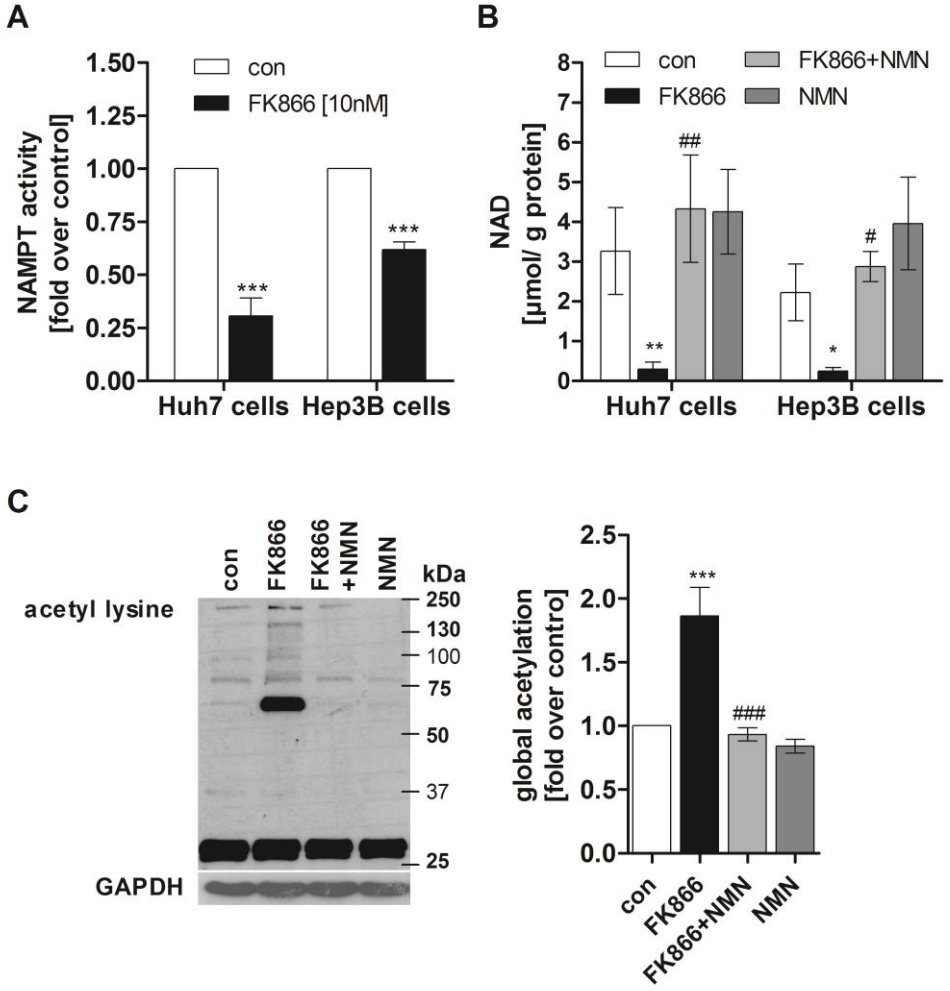


Figure 1. FK866 reduced NAMPT activity and NAD content and increased global acetylation of lysine residues. A) NAMPT activity and **B)** NAD content were measured after 24h and were normalized to total protein amount in each sample (n=3). **C)** Western Blot analysis of acetylated lysine residues in lysates of Hep3B cells treated with FK866 [10nM], a combination of FK866+NMN or NMN alone for 48h. GAPDH was used as loading control. Densitometric analysis of each lane was performed in four independent Western Blots (n=4). Cells stimulated with serum-free medium were used as control [con] and were set 1. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*$p<0.05$, **$p<0.01$, **$p<0.001$ compared to serum-free medium control; #$p<0.05$, ##$p<0.01$, ###$p<0.001$ compared to FK866 [10nM]).

NAMPT inhibition by FK866 reduced cell viability, induced energy stress and led to delayed cell death in human hepatocarcinoma cells

We could detect a decreased cell viability in hepatocarcinoma cells (-49.4±4.6% in Huh7 cells, -20.6±2.8% in Hep3B cells) (Fig.2A) after 24h of FK866 treatment. We wanted to investigate whether FK866-induced NAD depletion would result in a reduction of ATP generation and therefore would induce cellular energy stress in hepatocarcinoma cells. Time course studies revealed that ATP levels were lowered in Huh7 cells (-49.6±9.5%, $p<0.01$) and Hep3B cells (-61.1±6.8%, $p<0.001$) after 48h of treatment with 10nM FK866 (Fig.2B). The ATP levels further declined after 72h in Huh7 cells (-90.2±2.5%, $p<0.001$) and Hep3B cells (-91.1±1.5%, $p<0.001$) (Fig.2C). The co-administration of NMN could ameliorate ATP levels in Huh7 and Hep3B cells after 48 and 72h (Fig.2B, C).

After 72h, subsequent to the drop of NAD levels, the effects of FK866 on cell death became evident when measuring An^+/PI^+-stained cells. Hep3B cells, a p53-deficient cell line, already displayed an increase in An^+/PI^+ cells after 48h of FK866 treatment (+1.8-fold, $p<0.01$) (Supplement Fig.2A) indicating that FK866-induced cell death did not depend on p53 function. Huh7 cells treated with FK866 [10nM] for 72h showed a 1.5-fold increase in An^+/PI^+ cells compared to control cells ($p<0.05$) (Fig.2D) whereas the number of An^+/PI^+ Hep3B cells increased further (+3.0-fold, $p<0.01$). Co-stimulation with NMN ameliorated the induction of cell death in Huh7 cells ($p=0.09$) and completely rescued FK866-induced cell death in Hep3B cells ($p<0.01$) (Fig.2D).

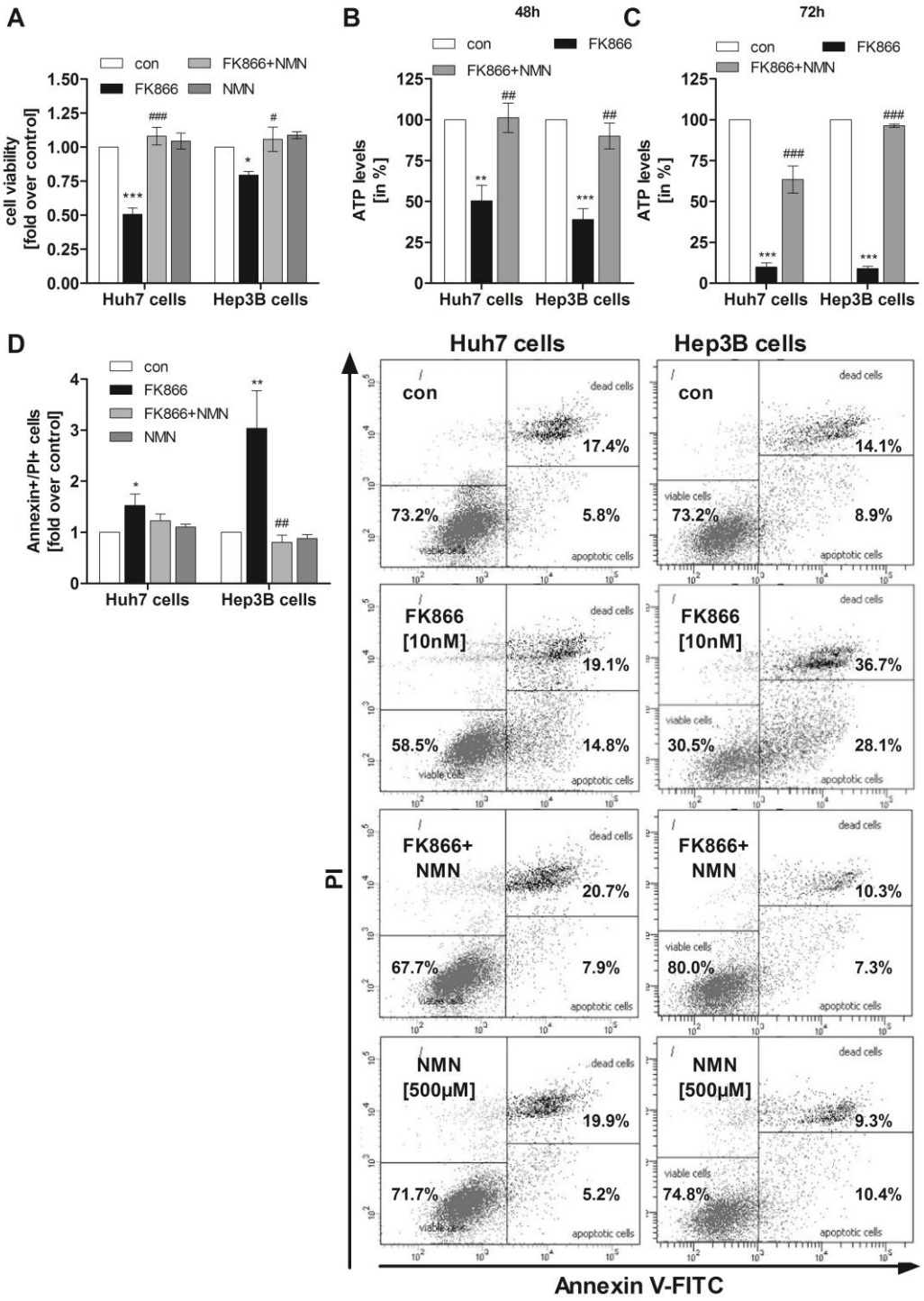


Figure 2. FK866-induced NAMPT inhibition reduced cell viability and ATP content and induced delayed cell death in hepatocarcinoma cells. A) Cell viability of Huh7 and Hep3B cells after 24h was measured using WST1-reagent (n=4). ATP content after **B)** 48h and **C)** 72h treatment with 10nM FK866 (n=3). Cells stimulated with serum-free medium were used as control [con] and were set 100%. **D)** AnnexinV-FITC/PI assay of Huh7 and Hep3B cells treated with FK866, FK866+NMN or NMN alone for 72 h (n=3). Cells stimulated with serum-free medium were used as control [con] and were set 1. Representative dot plots of the AnnexinV-FITC/PI staining in Huh7 and Hep3B cells are shown including the percentage of viable, An^+ and An^+/PI^+ - cells. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p<0.05, **p<0.01, **p<0.001 compared to serum-free medium control; #p<0.05, ##p<0.01, ###p<0.001 compared to FK866 [10nM]).

Dysregulation of the AMPK/mTOR signaling pathway in hepatocarcinoma cells compared to non-cancerous hepatocytes

Growing evidence suggests that mTOR and AMPK dysregulation play an important role in hepatocellular carcinogenesis [20;31]. Therefore, we compared the protein amount of mTOR and its downstream target p70S6 kinase and also AMPKα activation in non-cancerous primary human hepatocytes and hepatocarcinoma cells. An increased protein level of total mTOR and p70S6 kinase was found in hepatocarcinoma cells compared to non-cancerous hepatocytes (Fig.3A). In contrast, AMPK activation was enhanced in non-cancerous primary human hepatocytes (PHH) compared to Huh7 and Hep3B cells despite equal AMPKα total protein amount (Fig.3A). This suggests that mTOR signaling and AMPK activation are involved in metabolic adaptation of hepatocarcinoma cells and might be interesting targets for prevention of cancer cell growth.

FK866-induced energy stress activated AMPKα and led to inhibition of mTOR complex1 signaling in hepatocarcinoma cells

To test the efficacy of FK866-induced NAD depletion to activate AMPK and inhibit the mTOR signaling pathway, we measured the phosphorylation state of different members of the AMPK/mTOR complex1 cascade. FK866 treatment increased the phosphorylation of AMPKα at Thr172 (+3.3-fold, $p<0.01$) in hepatocarcinoma cells (Fig.3B). This was associated with a significant down regulation of phosphorylated mTOR (Ser2448) by -50.7±0.1% ($p<0.05$) and the phosphorylation of its down-stream target p70S6 kinase (by -94.7±2.4%, $p<0.001$) and 4E-BP1 (by -30.0±0.1%, $p<0.05$) indicating reduced protein synthesis and cell growth (Fig.3B).

Co-treatment with NMN [500μM] completely reversed the FK866-induced effects on AMPK activation and mTOR complex1 signaling inhibition suggesting that the NMN biosynthetic activity of NAMPT is relevant in mediating the effects of FK866. NMN alone had no impact on AMPK activation and mTORC1 signaling in hepatocarcinoma cells (Fig.3B). Non-cancerous human hepatocytes treated with equal amounts of FK866 for 48h did not show significant changes in AMPK activation and mTOR phosphorylation (Supplement Fig.1C) verifying their lower sensitivity to FK866.

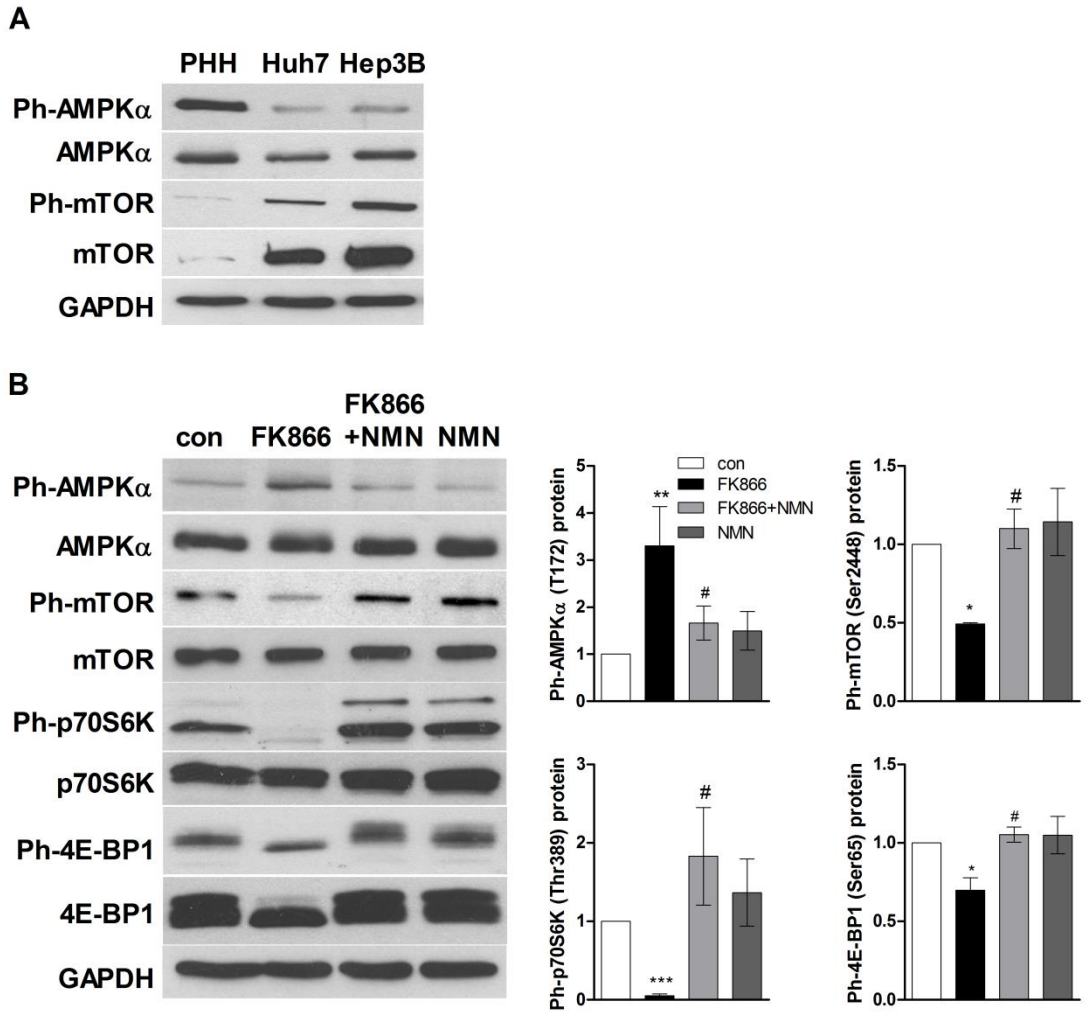


Figure 3. Expression of mTOR and AMPK in hepatocarcinoma cells and non-cancerous human hepatocyes and its regulation by FK866. A) Western blot analysis of AMPK and mTOR expression in lysates of non-cancerous, primary human hepatocytes (PHH), Huh7 and Hep3B cells (n=3). **B)** Western blot analysis of the AMPK/mTORC1 signaling pathway in lysates of Huh7 cells treated with FK866 [10nM], a combination of FK866 [10nM]+NMN [500μM] or NMN [500μM] alone for 48h (n=3). GAPDH was used as loading control. One representative blot out of 3 independent experiments is shown. Background-corrected densitometric values were normalized to control (serum-free medium). Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p<0.05, **p<0.01, **p<0.001 compared to serum-free medium control; #p<0.05, ##p<0.01, ###p<0.001 compared to FK866 [10nM]).

3.4 Discussion

During malignant transformation the cellular metabolism undergoes multiple molecular and metabolic adaptations to support cell growth and survival. NAD is a key determinant in cancer cell biology as it is essential for redox reactions and key component of signaling pathways that regulate transcription, DNA repair, apoptosis and metabolism [1]. In mammals, NAMPT is a main regulator of the intracellular NAD pool [2;3]. Here, we investigated whether or not the NAMPT inhibitor, FK866, would affect intracellular NAD

and ATP concentrations in hepatocarcinoma cells and consequently would be able to regulate the activity of the metabolic sensors AMPK and mTOR. Our study showed that FK866 rapidly reduced NAD levels in hepatocarcinoma cells and led to delayed ATP depletion which could be ameliorated by administration of NMN. Break down of ATP levels was associated with increased cell death. In contrast to another study [6], we demonstrated that FK866 reduced NAMPT activity, depleted NAD and ATP content and induced cell death in p53-deficient Hep3B cells suggesting that FK866-mediated cell death does not depend on functional p53. Our results are in line with a study performed in chronic lymphocytic leukemia cells [14]. In our study, especially Hep3B cells showed a high sensitivity to FK866 and an increased number of dead cells occurred already after 48h of FK866 treatment.

Interestingly, non-cancerous human hepatocytes subjected to the same FK866 treatment as hepatocarcinoma cells did not display reduced NAMPT activity and NAD content even at a FK866 concentration 10-fold of the EC_{50} (EC_{50} 8.2nM) indicating a lower sensitivity of non-cancerous cells to FK866. This has also been described for normal blood cells [6;7]. Therefore, FK866 represents an interesting compound in cancer cell therapy as it progressively exhausts NAD content in cells with a high NAD turnover that mainly rely on nicotinamide and the NAMPT-mediated NAD salvage pathway as source of NAD. Cancer cells have a significantly higher NAD turnover than normal cells to sustain their rapid proliferation, relative genomic instability, permanently ongoing DNA repair, increased aerobic glycolysis and increased activity of NAD-dependent deacetylases [1;12;13]. This is in line with results of our previous study showing that the expression of SIRT1, a NAD-dependent deacetylase, was significantly higher in hepatocarcinoma cells than in non-cancerous hepatocytes [9].

In this study we could demonstrate that NAMPT inhibition by FK866 led to a sharp decline of intracellular ATP levels and therefore induced energy stress. As a key physiological energy sensor, AMPK is a major regulator of cellular energy homeostasis that coordinates multiple metabolic pathways to balance energy supply [24]. Several studies have shown that AMPK activators exhibit inhibitory effects on cancer cell growth [32;33]. AMPK is known to phosphorylate and activate tuberous sclerosis complex (TSC)2, a negative regulator of mTOR [34]. Therefore, the AMPK/mTOR pathway serves as a signaling nexus for regulating cellular metabolism, energy homeostasis, and cell growth, and dysregulation of each pathway may contribute to the development of HCC [20;26]. Since the discovery that the mTOR pathway is hyperactivated in many cancers including HCC [25;26;31;35], there is a great interest in finding molecular pathways and novel compounds

that target AMPK/mTOR signaling as novel treatment option for HCC. We could show that components of the mTORC1 cascade were significantly higher expressed in hepatocarcinoma cells than in non-cancerous hepatocytes. Additionally, our data revealed that the activation of AMPK was significantly decreased in hepatocarcinoma cells. Reduced AMPK activity has also been detected in primary human breast cancer [36] and lymphoma [21] cells. Thus, a dysregulated AMPK activity may represent an important regulatory step during tumor initiation and progression, allowing cancer cells to gain a metabolic growth advantage by enhancing aerobic glycolysis (Warburg effect) [21]. We made the intriguing discovery that FK866 acts as an AMPK activator in cancer cells potentially through its ability to induce cellular energy stress. Activation of AMPK was associated with a down regulation of the mTORC1 pathway. All FK866 induced effects could be completely reversed by NMN suggesting that these effects were mediated by NAD. mTORC1 inhibition led to decreased activation of its two downstream targets, 70S ribosomal protein S6 kinase (p70S6K) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1). p70S6K and 4E-BP1 are major regulators of protein translation and cellular growth [35]. This contradicts a study performed in neuronal cells where FK866 or a NAMPT knock down was shown to reduce AMPK activation [37]. However, this can be explained by the use of non-cancerous neuronal cells compared to cancer cells in our study.

In summary, our study showed the importance of the NAMPT-mediated NAD salvage pathway for energy homeostasis in hepatocarcinoma cells. Furthermore, FK866-induced NAMPT inhibition led to activation of AMPK and inhibition of mTOR signaling suggesting a putative use of FK866 alone or as a chemotherapeutic sensitizing drug to reduce cancer cell growth. In every case of potential therapeutic use, administration of NMN as antidote may be useful to modulate or counteract FK866 toxicity. Only early stages of HCC are curable with today's treatment protocols, therefore new therapeutic strategies are urgently needed and NAMPT inhibition represents a potential novel treatment approach.

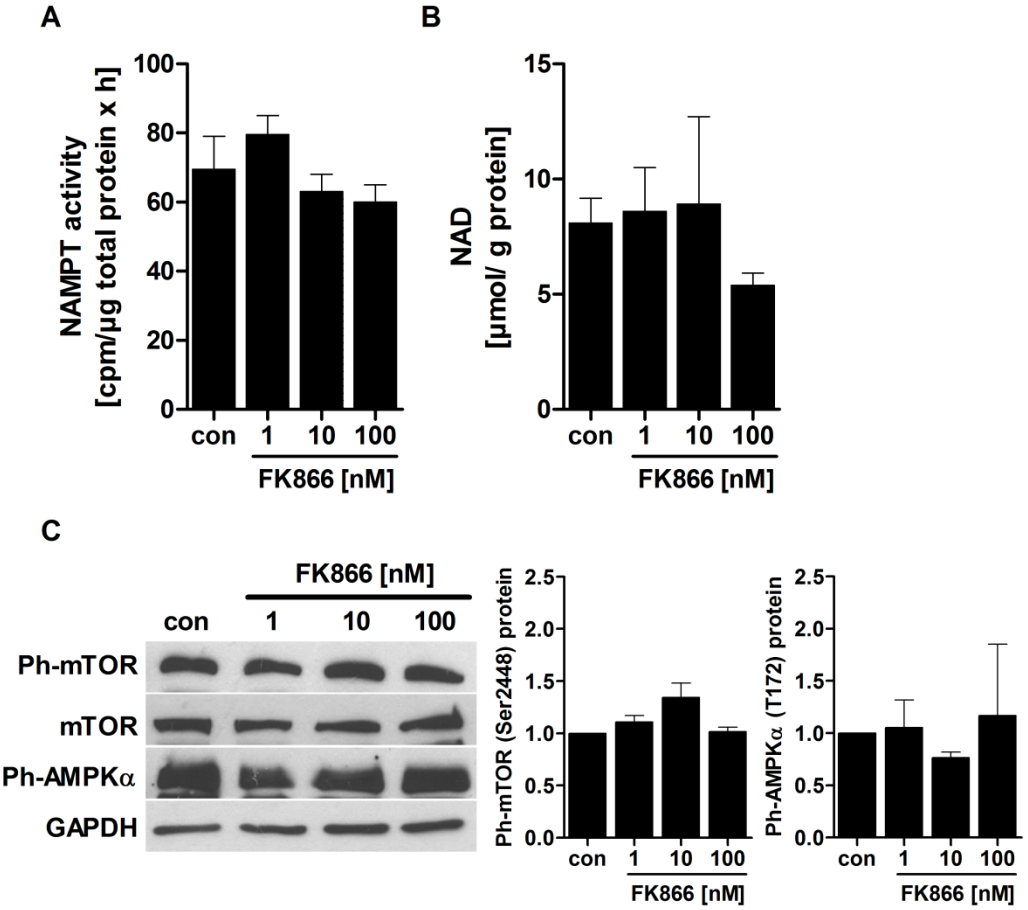
3.5 Acknowledgments

Primary human hepatocytes were kindly provided by the “Virtual Liver Network”, a major national initiative on Systems Biology of the Liver. This work was supported by the German Federal Ministry of Education and Research (BMBF), “Virtual Liver Network”, grant FKZ 0315753 to TSW. We thank our technician Sandy Richter for excellent work. We also gratefully thank Prof. Dr. Kurt Engeland (Molecular Oncology, Faculty of

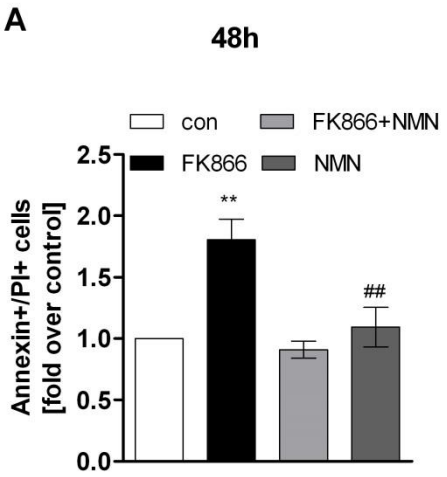
Medicine, University of Leipzig) for providing Huh7 cells and Prof. Dr. Sven-Erik Behrens (Institute of Biotechnology, Martin-Luther-University Halle-Wittenberg) for providing Hep3B cells.

Detailed author contributions can be found at the end of this work.

3.6 Supplementary Material



Supplement Figure 1. Effects of FK866 on NAMPT activity, NAD content and AMPKα/mTOR activity in non-cancerous human hepatocytes. Non-cancerous primary human hepatocytes were stimulated with different doses of FK866 [1, 10, 100nM] in serum-free medium for 48h. **A)** NAMPT activity and **B)** NAD content were measured and normalized to total protein amount in each sample. **C)** Western blot analysis of mTOR expression and AMPKα activation were performed using specific antibodies against phospho-mTOR (Ser2448), total mTOR and phospho-AMPKα (Thr172). Background-corrected densitometric values were normalized to control (serum-free medium). Data are represented as mean± SEM of two independent experiments (n=2) and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test.



Supplement Figure 2. FK866 induced cell death in Hep3B cells after 48h. A) AnnexinV-FITC/PI assay of Hep3B cells treated with FK866 [10nM], FK866 [10nM] + NMN [500μM] or NMN alone for 48 h (n=3). An^+ and An^+/PI^+- stained cells were considered as dead cells and are depicted in the graph. Cells stimulated with serum-free medium were used as control [con] and were set 1. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (**$p<0.001$ compared to serum-free medium control; ##$p<0.01$ compared to FK866 [10nM]).

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CHAPTER 4

Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes

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Resveratrol is reported to possess chemotherapeutic properties in several cancers. In this study, we wanted to investigate the molecular mechanisms of resveratrol-induced cell cycle arrest and apoptosis as well as the impact of resveratrol on NAMPT and SIRT1 protein function and asked whether there are differences in hepatocarcinoma cells (HepG2, Hep3B cells) and non-cancerous primary human hepatocytes. We found a lower basal NAMPT mRNA and protein expression in hepatocarcinoma cells compared to primary hepatocytes. In contrast, SIRT1 was significantly higher expressed in hepatocarcinoma cells than in primary hepatocytes. Resveratrol induced cell cycle arrest in the S- and G2/M- phase and apoptosis was mediated by activation of p53 and caspase-3 in HepG2 cells. In contrast to primary hepatocytes, resveratrol treated HepG2 cells showed a reduction of NAMPT enzymatic activity and increased p53 acetylation (K382). Resveratrol induced NAMPT release from HepG2 cells which was associated with increased NAMPT mRNA expression. This effect was absent in primary hepatocytes where resveratrol was shown to function as NAMPT and SIRT1 activator. SIRT1 inhibition by EX527 resembled resveratrol effects on HepG2 cells. Furthermore, a SIRT1 overexpression significantly decreased both p53 hyperacetylation and resveratrol-induced NAMPT release as well as S-phase arrest in HepG2 cells. We could show that NAMPT and SIRT1 are differentially regulated by resveratrol in hepatocarcinoma cells and primary hepatocytes and that resveratrol did not act as a SIRT1 activator in hepatocarcinoma cells.

4.1 Introduction

Resveratrol, a dietary polyphenol, is reported to possess both chemopreventive and chemotherapeutic properties in several cancers [1]. In 1997, Jang and colleagues published a seminal paper reporting that resveratrol is able to inhibit carcinogenesis in all three stages (initiation, promotion and progression) [2]. Resveratrol was shown to inhibit cell proliferation, induce apoptosis and cell cycle arrest in different cancer types and cancer cell lines [3–9]. However, only one study compared the apoptotic effects of resveratrol on cancer and normal cells. Baarine *et al.* found apoptotic effects in murine tumoral cardiac cells which were absent in normal cardiomyocytes [8]. The molecular mechanisms are currently not completely understood. SIRT1 has originally been described as a target of resveratrol [10] although some of the data are still controversial, especially concerning resveratrol acting as SIRT1 activator in cancer cells [11–13]. SIRT1 belongs to the NAD (Nicotinamide adenine dinucleotide) dependent histone deacetylases, called sirtuins. SIRT1 is involved in many cellular pathways, such as cellular survival, apoptosis, cellular stress response and energy metabolism. An increased expression of SIRT1 has been reported in a variety of human cancers, including prostate, ovarian, gastric and colorectal cancer. The role of SIRT1 in tumorigenesis is still controversially discussed. SIRT1 has been shown to act as both tumor promoter and tumor suppressor [14,15]. SIRT1 was shown to deacetylate the tumor-suppressor protein p53 on lysine residue 382 leading to its inhibition and subsequent tumorigenesis [16,17]. Thus, the inhibition of SIRT1 would induce cell death of cancer cells by activating and acetylating p53.

It is known that cancer cells have increased energy demands because of their rapid cell proliferation and increased DNA repair [18]. NAD is required for both processes [19] and regulates crucial biological processes, including transcription, cell cycle progression, DNA repair and metabolic pathways [20,21]. Therefore, cancer cells have a higher rate of NAD turnover than normal cells. The regeneration of intracellular NAD pools is regulated by NAMPT (Nicotinamide phosphoribosyltransferase). NAMPT can be found intracellularly (iNAMPT) and extracellularly (eNAMPT). However, neither structural differences between these forms nor the mechanism of NAMPT secretion are known so far. As an intracellular protein, NAMPT catalyses the rate-limiting step in the NAD salvage pathway starting from nicotinamide and yielding nicotinamide mononucleotide (NMN) which is then converted to NAD [22–25]. Some cancer cells maintain intracellular NAD levels by overexpressing NAMPT which has been shown in different cancer types, such as colorectal and breast cancer [26–28]. The expression and regulation of intracellular

NAMPT in hepatocarcinoma cells has not been characterized so far. NAMPT inhibition by its highly specific inhibitor FK866 induces apoptosis and/or autophagy in tumor cells [29–32]. Moreover, previous studies pointed out that inhibition of NAMPT enzymatic activity by FK866 or inhibition of SIRT1 activity decreased proliferation and triggered cell death in cancer cells which was associated with increased acetylation of p53 (K382) [16,17,33,34].

Here we investigated the molecular mechanisms of resveratrol-induced apoptotic effects on hepatocarcinoma cells and non-cancerous human hepatocytes and asked whether NAMPT and SIRT1 are differentially regulated in hepatocarcinoma cells and non-cancerous human hepatocytes.

4.2 Material and Methods

Ethics Statement

Non-cancerous primary human hepatocytes were supplied by the "virtual liver" program (German Federal Ministry of Education and Research) and the non-profit foundation HTCR, including the informed patient's consent. The use of human hepatocytes for research purposes was approved by the local ethics committee of the Charité University Berlin. Written informed consent was obtained from all patients. The Charité University Berlin institutional review board specifically approved this study.

Material

Cell culture media, supplements and antibiotics were obtained from PAA (Cölbe, Germany) or Invitrogen (Karlsruhe, Germany). Resveratrol (*trans* isomer), nicotinamide and camptothecin were purchased from Sigma-Aldrich (Munich, Germany). FK866 was kindly provided by TopoTarget A/S, Copenhagen, Denmark. EX527 was obtained from Cayman Chemical (Ann Arbor, USA), InSolution Trichostatin A (TSA) and etoposide were purchased from Merck Millipore (Darmstadt, Germany). Flag-SIRT1 expression vector was obtained from Addgene (Addgene plasmid 1791) [35].

Cell culture

HepG2 cells were purchased from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell cultures) and Hep3B cell were kindly provided by Prof. Dr. Kurt Engeland (Molecular Oncology, Medical School, University of Leipzig). Cells were maintained in MEM medium supplemented with 10% fetal bovine serum (FBS) and 2

mmol/L glutamine and 100 IU penicillin and 100 μg/mL streptomycin. Primary human hepatocytes were isolated and cultured essentially as described [36]. Cells were seeded in Williams' Medium E containing 2 mmol/L glutamine, 10^{-7} mol/L dexamethansone, 100 IU penicillin and 100 μg/mL streptomycin and 10% FBS. All cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Cell treatments

Resveratrol was dissolved in 100% ethanol to create a stock solution of 100 mM. Cells were stimulated with 10/25/50/100 μM resveratrol and the equivalent amount of solvent control (ethanol) to exclude solvent-mediated effects. To inhibit SIRT1 and deacetylases other than histone deacetylases class III, we used the compound EX527 [20 μM], a cell-permeable selective inhibitor of SIRT1 dissolved in DMSO [37] and 1 μM of TSA which were added to the incubation medium.

Measurement of cell viability and apoptosis

To investigate the effects on proliferation and cell viability, we used the commercial Cell Proliferation Reagent WST-1 (Roche, Grenzach-Wyhlen, Germany) and measured absorbance at 450 nm. To evaluate the effects of resveratrol on apoptosis the number of apoptotic cells was measured by flow cytometry using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, Franklin Lakes, USA). Adherent and floating cells were used. 5-10 μL of Annexin V-FITC (An) and 2 μL of propidium iodide (PI) were added to the cell suspension. Samples were analysed using a Beckton-Dickinson FACS LSRII. As positive control, apoptosis was induced via camptothecin [2 μM] and etoposide [85 μM] for 24 h. An+ and double-stained An+/PI+ cells were considered apoptotic. To exclude cytotoxic effects of resveratrol, we used supernatant of HepG2 cells and primary human hepatocytes to measure the release of the enzyme, adenylate kinase, from damaged cells. Therefore, we used the ToxiLight™ Non-destructive Cytotoxicity BioAssay Kit (Lonza, Cologne, Germany).

Cell cycle distribution analysis

PI staining was used to analyse DNA content and cell cycle distribution. After cell treatment, adherent and floating cells were harvested and fixed with 2 mL of 70% ethanol (4ºC). The cell pellet was resuspended in 50 μL PBS with 3.3 μL RNase A [30 mg/mL], 450 μL FACS-buffer (PBS+ 2% FBS) and PI [50 μg/mL] were added to the flow

cytometry tubes. Cells were analysed using a Beckton-Dickinson FACS LSRII by measuring the PI signal in the FL2 channel.

Reverse Transcription-Quantitative Real-time PCR (RTqPCR)

To measure mRNA expression, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using 200 U M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) per 500 ng or 1 μg total RNA with random hexamer [p(dN)6] primers. mRNA expression was quantified by real-time PCR with TaqMan probe based (Eurogentec, Cologne, Germany) or SYBR green based (Primerdesign, Southampton, UK) gene expression assay on the ABI 7500 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). The housekeeping genes *TATA-box-binding protein* (TBP), *hypoxanthine phosphoribosyltransferase* (HPRT) or beta-*ACTIN* were quantified simultaneously. Sequence information of primers and probes are given in Table 1. For standardization of gene expression, the target gene amount was normalized to the mean of the housekeeping gene expression in each sample.

Table 1. Sequences of Primer and Probes used for *real-time* PCR (TaqMan®).

Target	Forward Primer	Reverse Primer	Probe
NAMPT	GCA GAA GCC GAG TTC AAC ATC	TGC TTG TGT TGG GTG GAT ATT G	TGG CCA CCG ACT CCT ACA AGG TTA CTC AC
beta-ACTIN	CGA GCG CGG CTA CAG CTT	CCT TAA TGT CAC GCA CGA TTT	ACC ACC ACG GCC GAG CGG
TBP	TTG TAA ACT TGA CCT AAA GAC CAT TGC	TTC GTG GCT CTC TTA TCC TCA TG	AAC GCC GAA TAT AAT CCC AAG CGG TTT G
HPRT	GGC AGT ATA ATC CAA AGA TGG TCA A	GTC TGG CTT ATA TCC AAC ACT TCG T	CAA GCT TGC TGG TGA AAA GGA CCC C
p21	CGAAGTCAGTTCCTTGT GGAG	CATGGGTTCTGACGGAC AT	-

*NAMPT (*nicotinamide phosphoribosyltransferase, also known as PBEF, visfatin); *p21*; housekeeping genes *beta-ACTIN*, *TBP* (TATA-box-binding protein) and *HPRT* (hypoxanthine phophoribosyltransferase).

Protein extraction and immunoblotting

For protein analyses, cells were lysed in modified RIPA buffer (50 mM TrisHCl pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 1x Roche complete proteases inhibitor cocktail; 1 mM EDTA; 1 mM sodium orthovanadate; 1 mM sodium fluoride; 5 mM nicotinamide; 5 μM TSA, 1mM sodium butyrate) and separated by SDS-PAGE (8-15%). Protein concentration of lysates was measured by BCA protein assay (Pierce, Thermo Scientific). After transfer to nitrocellulose membranes (Millipore, Bedford, MA, USA), blots were blocked with 5% (w/v) non-fat dry milk in 1x TBS buffer containing 0.1% Tween 20 (TBS-T). Primary antibodies used for immunoblotting included anti-NAMPT clone OMNI 379 (1:5000) (Cayman Chemical, Ann Arbor, MI, USA), anti- acetylated p53 (K382) (1:1000), anti-p53 (1:1000), anti-p53 (1C12) (1:1000), anti-phospho-p53 (Ser15) (1:1000), anti-SIRT1 (D379) (1:1000), anti-p21 (1:1000), anti-Bax (1:1000), anti-caspase3 (1:500), anti- cleaved caspase3 (1:500) (Cell Signaling, Beverly, MA, USA) and anti-GAPDH (MerckMillipore, Schwalbach, Germany). Secondary antibodies were purchased from DAKO (Hamburg, Germany). Immunoblotting for GAPDH was performed to verify equivalent amounts of loaded protein. Detection was performed using enhanced chemiluminescence. Densitometric analysis was performed using ImageJ 1.41 Software (NIH, USA).

Measurement of NAMPT release

NAMPT concentration in supernatants of HepG2 cells and primary hepatocytes was quantified using the human extracellular NAMPT/PBEF/Visfatin ELISA Kit (AdipoGen Inc., Seoul, South Korea), respectively, according to manufacturer's instructions. NAMPT concentration was normalised to the corresponding total protein amount in each sample. For semiquantitative measurements, NAMPT levels were detected by using supernatant of cultured cells for Western Blot analysis.

NAMPT enzymatic activity

NAMPT activity was measured by the conversion of ^{14}C- labelled nicotinamide to ^{14}C-NMN using a method previously described [38]. For preparation of lysates, cells were harvested and resuspended in 100 μL of 0.01 mol/L sodium phosphate buffer, pH 7.4, frozen at –80°C for 24 h and thawed at room temperature. Cell debris was removed by centrifugation at 23,000 rcf, 90 min at 0°C. Protamine sulphate solution (1% in NaHPO4 buffer) was added to the supernatant (70 μL/mL supernatant) to precipitate DNA by

incubation on ice for 15 min. After centrifugation at 23,000 rcf, 30 min at 0°C, aliquots of the supernatant were stored at –80°C. Lysates (50μg) were added to 50 μL reaction mix (50 mmol/L TrisHCl; 2 mmol/L ATP; 5 mmol/L MgCl2; 0.5 mmol/L PRPP; 6.2 μmol/L ^{14}C-nicotinamide; American Radiolabelled Chemicals, St. Louis; MO, USA) and incubated at 37°C for 1h. Optimal conditions for the NAMPT activity assay (amount of total protein, incubation time, pH value) were determined (Fig.S1A,B,C). For measuring extracellular NAMPT activity we used supernatant of HepG2 cells and concentrated it 80-fold using Amicon Ultra Centrifugal Filter Units (Ultracel-50k) (Millipore). Then, 10μl of concentrated supernatant was used for the enzyme assay reaction mix and incubated for 2h at 37°C. The NAMPT enzymatic reaction was terminated by mixing with 2 mL of acetone. The mixture was then transferred onto acetone-pre-soaked glass microfiber filters (GF/A Ø 24 mm; Whatman, Maidstone, UK). After rinsing with 2x 1 mL acetone, filters were dried, transferred into vials with 6 mL scintillation cocktail (Betaplate Scint, PerkinElmer, Waltham, MA, USA) and radioactivity of ^{14}C-NMN was quantified in a liquid scintillation counter in counts per minute (cpm) (Wallac 1409 DSA, PerkinElmer). NAMPT activity was normalised to total protein concentration as measured by the BCA protein assay. The validity of the assay was evaluated by adding the specific NAMPT inhibitor, FK866 (Fig.S1C). FK866 induced a dose-dependent decrease in NAMPT activity with an IC_{50} value of 8.2 nM.

NAD Measurements

Concentrations of NAD from whole-cell extracts were quantified by High-performance liquid chromatography *(*HPLC) and the NAD/NADH assay kit (EnzyChrom NAD/NADH Assay Kit, Biotrend, Köln, Germany), applied according to manufacturer's instructions. HPLC analysis was performed with Chromaster Purospher STAR RP-18 endcapped 3 μm Hibar RT 150-3 HPLC column (Merck). Briefly, cultured cells were extracted in 1M $HClO_4$ and neutralized in 3M K_2CO_3 on ice as described previously [39]. After centrifugation for 10 min at 18,000 rcf (4°C), the supernatant was filtered and loaded onto the column. For NAD measurement, the HPLC was run at a flow rate of 0,4 ml/min with 100% buffer A from 0-5 min, a linear gradient to 95% Buffer A/5% Buffer B (100% methanol) from 5-6 min, 95% Buffer A/5% Buffer B from 6-11 min, a linear gradient to 85% Buffer A/15% Buffer B from 11-12 min, 85% Buffer A/15% Buffer B from 12-16 min, and a linear gradient to 100% Buffer A from 16-17 min. NAD was eluted as a sharp peak at 15 min and quantitated based on the peak area compared to a standard curve and normalised to protein content of cultured cells.

Plasmid transfection

Transfection was conducted using NEON Transfection System (100µl Kit, invitrogen) according to the manufacturer's manual. Briefly, HepG2 cells were splitted 1:3 one day before transfection. Cells were transiently transfected with pECE-Flag-SIRT1 (2µg DNA/ 0.5 x 10^6 cells) or the empty vector (mock-control). After 24 h of transfection, medium was changed for a further 24h resveratrol-containing medium at 37 C.

Statistical analyses

Data are presented as mean ± SEM. Data were analysed for statistical significance by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Differences between two groups were evaluated using unpaired Student's *t*-test. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, USA). The level of significance for all comparisons was set at $p < 0.05$.

4.3 Results

NAMPT and SIRT1 are differentially expressed in hepatocarcinoma cells and primary human hepatocytes

The expression levels of NAMPT and SIRT1 were evaluated using qPCR and Western Blot analysis. Our data revealed that NAMPT expression is lower in HepG2 (-75.6%±5.2%) and Hep3B cells (-84.6%±0.5%) compared to non-cancerous primary human hepatocytes (Fig.1A,B). In contrast, the NAD-dependent deacetylase SIRT1 is significantly higher expressed in both cancer cell lines compared to primary human hepatocytes (HepG2 cells 2.8-fold, Hep3B cells 2.5-fold) (Fig.1A,B). Intracellular NAD levels in HepG2 cells and primary hepatocytes were not significantly different (HepG2 cells 1.9±0.3 µmol NAD/g protein compared to 1.7±0.3 µmol NAD/g protein in primary human hepatocytes) (Fig.1C, left panel).

A comparison of the NAMPT enzymatic activity in HepG2 cells and primary human hepatocytes showed a 3-fold higher ($p < 0.05$) enzymatic activity of NAMPT in HepG2 cells (57.2±7.7 cpm/µg protein x h) than in primary human hepatocytes (19.3±3.8 cpm/µg protein x h) (Fig.1C, middle panel). Additionally, we measured higher eNAMPT levels in the supernatant of primary human hepatocytes (3.2±0.3 ng/mg protein) than in HepG2 cells (0.4±0.2 ng/mg protein) (Fig.1C, right panel).

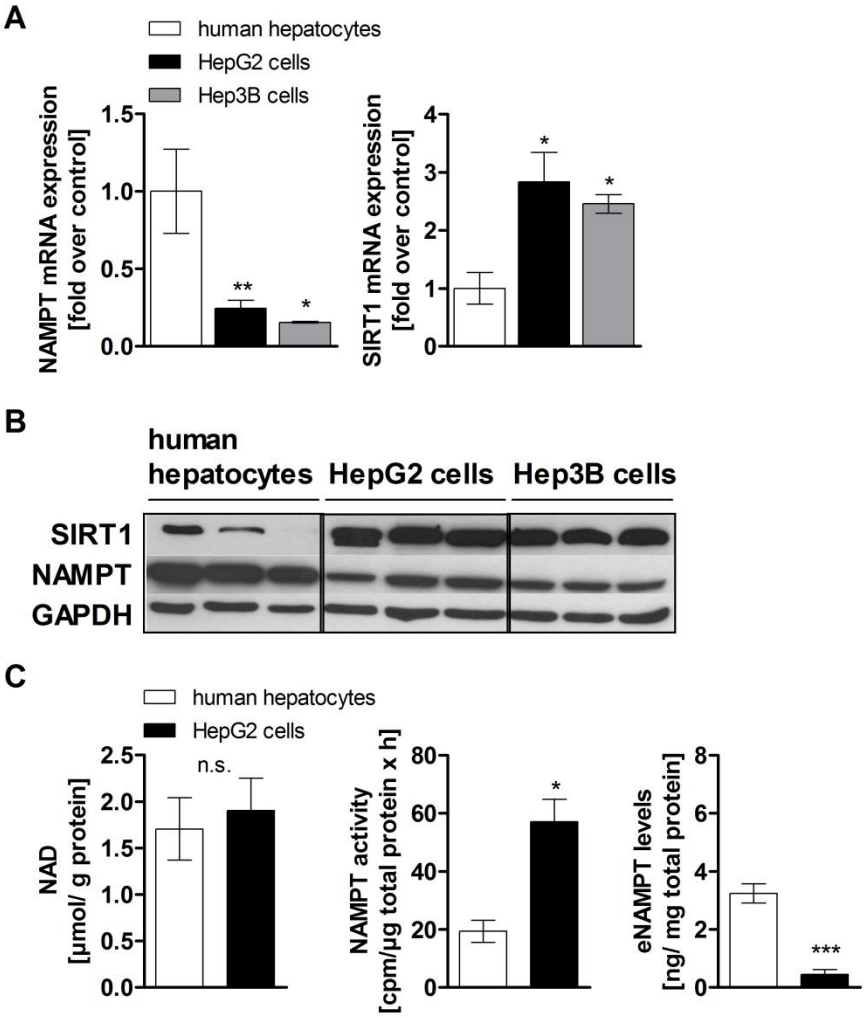

Figure 1. NAMPT and SIRT1 expression in hepatocarcinoma cells and primary human hepatocytes. A) mRNA expression and **B)** protein expression of NAMPT and SIRT1 in primary human hepatocytes (n=7), HepG2 cells (n=8) and Hep3B cells (n=3). Representative Western Blot is shown out of three independent experiments. Measurement of **C)** intracellular NAD levels (left panel, primary hepatocytes n=4, HepG2 cells n=6), basal NAMPT enzymatic activity (middle panel, primary hepatocytes n=3, HepG2 cells n=4) and extracellular NAMPT (eNAMPT) levels (right panel, primary hepatocytes n=3, HepG2 cells n=6) in primary human hepatocytes and HepG2 cells. Data are shown as mean± SEM. Difference between two groups was evaluated using unpaired Student's *t*-test (*p< 0.05, **p< 0.01, ***p< 0.001).

Resveratrol induces cell cycle arrest and apoptosis in hepatocarcinoma cells

Resveratrol has been shown to induce growth arrest and apoptosis in many different cancer cell lines. In the present study, we wanted to investigate whether the effects of resveratrol are p53-dependent. Therefore, we used HepG2 cells, known to be p53 wild-type, Hep3B cells, a p53 deficient cell line due to a deletion of the p53 gene, and primary human hepatocytes as non-cancerous hepatocyte control. Cells were treated with resveratrol as described above. After 24 h, hepatocarcinoma cells showed a dose-dependent decrease in viability (Fig.2A). Resveratrol [100 μM] markedly decreased cell viability by 45.8±2.7% ($p< 0.05$) in HepG2 cells and by 63.7±3.4% ($p<0.01$) in Hep3B cells (Fig.2A). Primary human hepatocytes treated with the same concentrations of resveratrol exhibited no significant changes in viability (Fig.2A).

To investigate the cause of cell viability reduction by resveratrol, we analysed cell cycle distribution. As shown in Fig.2B, resveratrol [25, 50 μM] caused an increase of cells in the S-phase ([con] 4.7±0.6%, [25 μM] 21.7±5.1%, [50 μM] 17.0±2.6%, $p< 0.05$) and in the G2/M-phase ([con] 13.7±1.8%, [25 μM] 23.9±4.2%, [50μM] 27.0±6.1%, $p< 0.05$) and a corresponding decrease of cells in the G1-phase. The cell cycle distribution was not significantly modified in p53-deficient Hep3B cells (Fig.S2A), which indicates that the resveratrol-induced cell cycle arrest is mediated by a functional p53.

However, in both hepatocarcinoma cell lines apoptotic mechanisms were activated. As indicated in Fig.2C, stimulation with increasing concentrations of resveratrol led to a dose-dependent increase in the number of apoptotic cells in HepG2 (Fig.2C,D) and Hep3B cells (Fig.2C). At 100 μM resveratrol, the percentage of apoptotic cells in HepG2 cells and Hep3B cells increased to 40.6±5.6% ($p< 0.01$) and to 32.2±3.7% ($p< 0.05$), respectively.

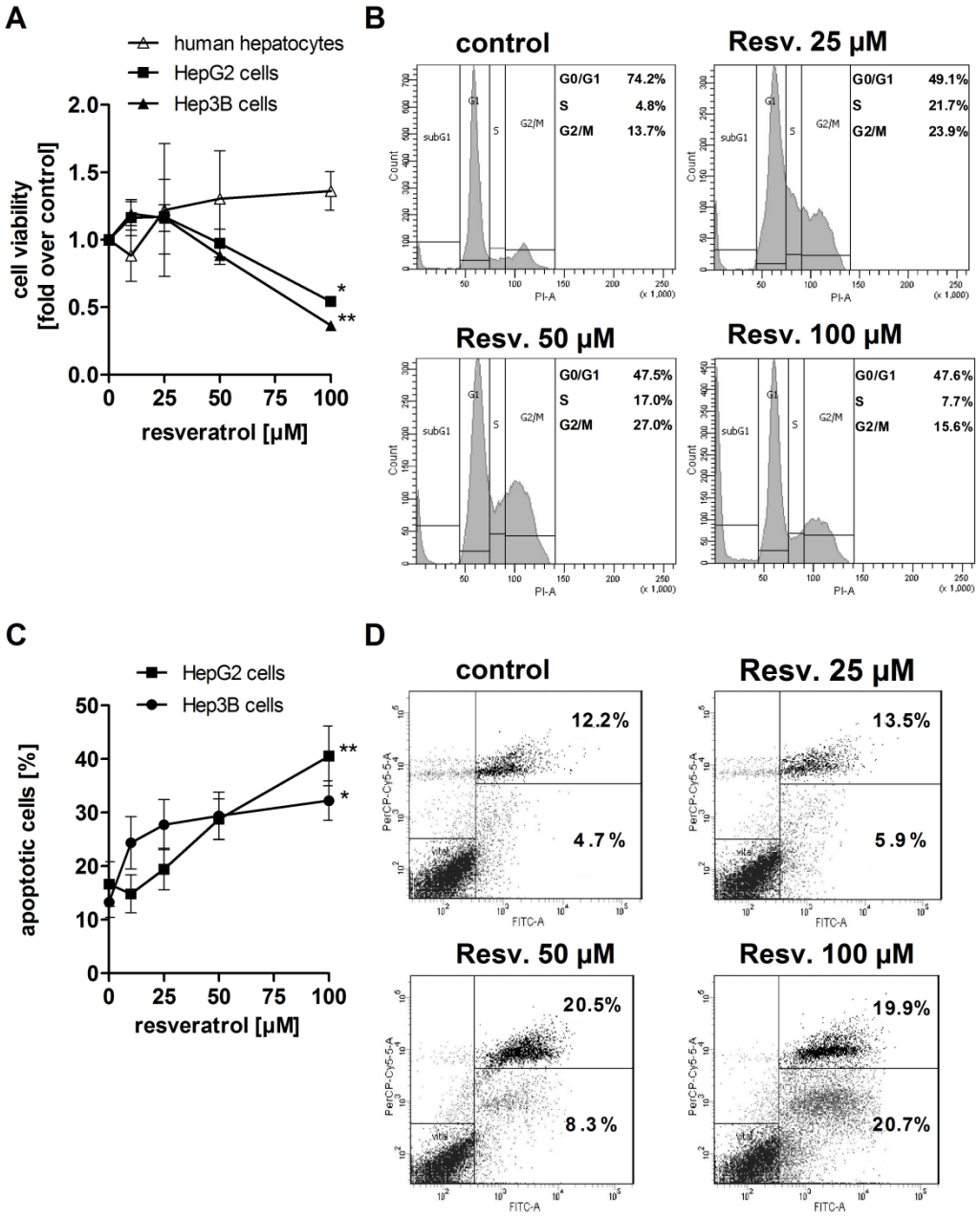

Figure 2. Resveratrol reduces cell proliferation and induces cell cycle arrest and apoptosis in hepatocarcinoma cells which is absent in primary human hepatocytes. Cell viability of **A)** primary human hepatocytes (n=2), HepG2 and Hep3B cells (n=3) after stimulation with resveratrol for 24 h. Data were normalised to serum-free medium control which was set 1. **B)** Cell cycle distribution of HepG2 cells treated with resveratrol for 24h. A representative result is shown out of three independent experiments. A representative dot plot is given in Fig.S2B. **C)** Annexin V/PI apoptosis assay of HepG2 (n=3) and Hep3B cells (n=3) treated with resveratrol for 24h. **D)** A representative dot plot of the Annexin/PI staining in HepG2 cells is shown including the mean percentage of An+ and double An+/PI+ cells of three independent experiments. Data are shown as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p< 0.05; **p< 0.01 compared to serum-free medium).

p53 and caspase-3 are involved in resveratrol-mediated apoptotic effects

In HepG2 cells, resveratrol increased the phosphorylation of p53 at residue serine 15 in a dose-dependent manner (Fig.3A). At high concentration of resveratrol [100μM], we found increased cleavage of caspase-3 (Fig.3A). The activation of caspase-3 by resveratrol was also increased in p53-deficient Hep3B cells even at lower concentration [25, 50μM] (Fig.3B). These results indicate that resveratrol induces caspase-3 activation in a p53-independent manner. Then, we stimulated primary human hepatocytes with the same concentrations of resveratrol and found no induction of apoptosis (Fig.3C) or cytotoxicity (Fig.S3A).

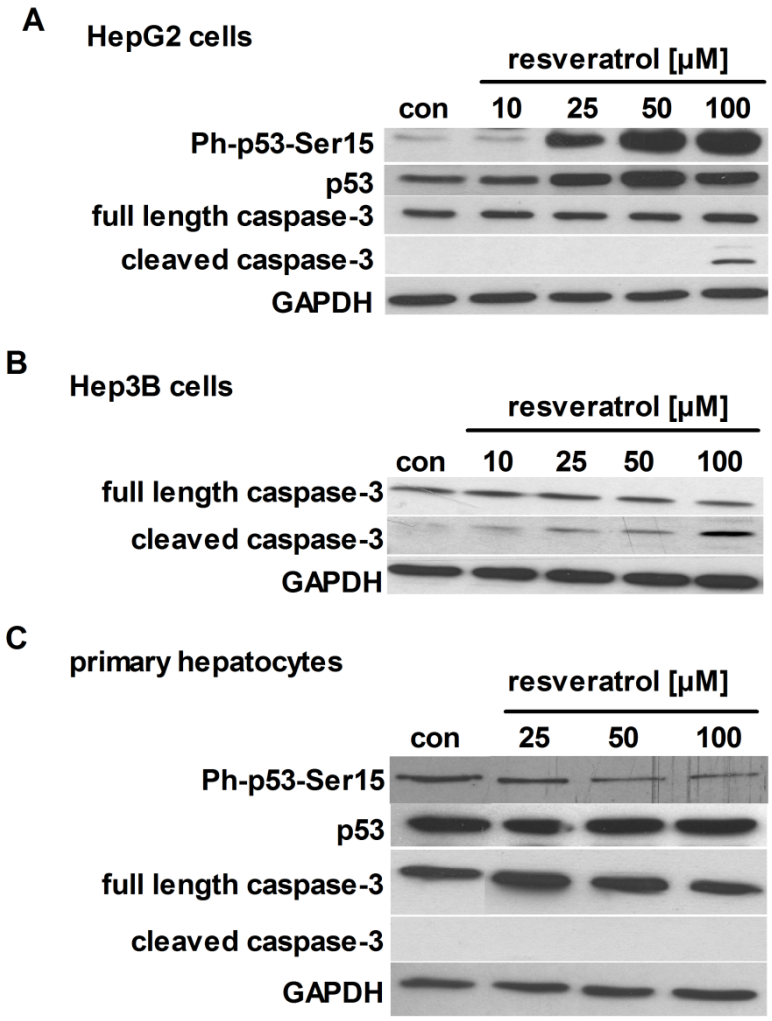

Figure 3. Resveratrol activates apoptotic mechanisms in hepatocarcinoma cells. Cells were treated with resveratrol or serum-free medium (con) for 24h. Activation of p53 through phosphorylation at serine residue 15 and cleavage of caspase-3 in **A)** HepG2 cells, **B)** Hep3B cells and **C)** primary human hepatocytes were analysed by Western Blot. GAPDH was used as loading control. One representative blot out of at least 3 independent experiments is shown.

Inhibition of NAMPT and SIRT1 activity in hepatocarcinoma cells induces growth arrest and apoptosis

Several studies have shown that the NAD metabolism is essential for cancer cell survival and proliferation [40–42]. However, little is known about the effects of resveratrol on NAMPT and SIRT1 activity in hepatocarcinoma cells. Human SIRT1 targets and deacetylates the p53 tumor suppressor protein [16,17,34]. Therefore, we investigated whether a specific inhibition of NAMPT and SIRT1 would affect cell survival and apoptotic mechanisms. We used the specific NAMPT inhibitor FK866 and the SIRT1 inhibitor EX527 [34]. FK866 increased p53 acetylation (K382) (Fig.4A) and reduced HepG2 cell viability after 48h (Fig.4B). Cells treated with the SIRT1 inhibitor, EX527, showed also increased p53 acetylation (K382), enhanced expression of p53 downstream target p21/WAF1/Cip1 and activation of caspase-3 (Fig.4C).

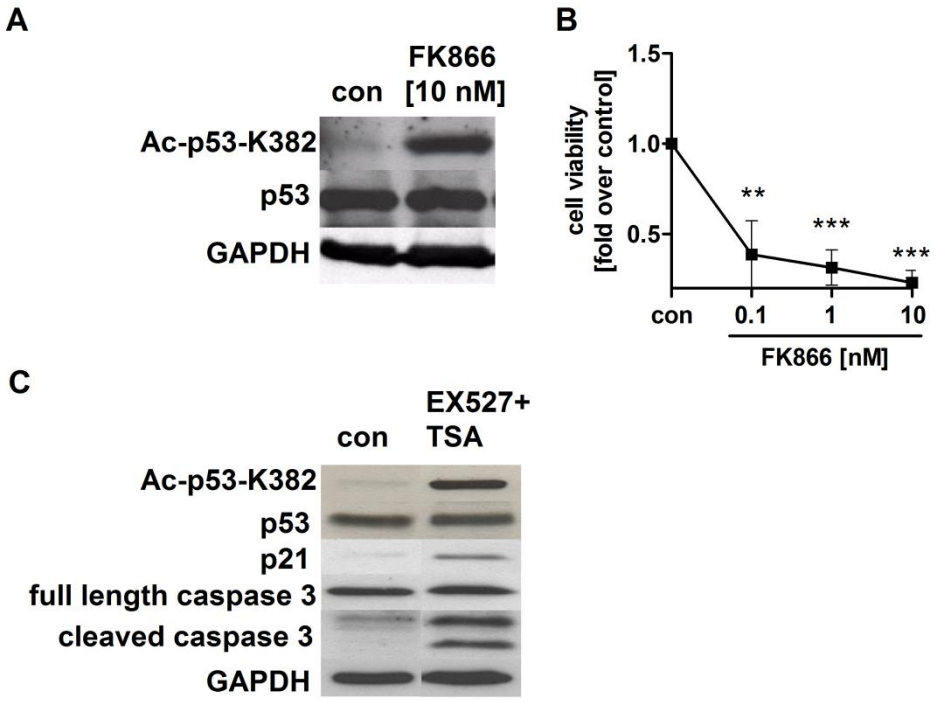

Figure 4. Effects of FK866 and EX527 on p53 acetylation and cell viability in HepG2 cells. Cells were stimulated with FK866 [10 nM] or EX527+TSA [20μM EX527+ 1μM TSA] in serum-free medium (con). Cells treated with **A)** FK866 and expression of acetylated p53 (K382) after 24 h. **B)** Cell viability of HepG2 cells after stimulation with FK866 for 48h measured by WST-1 assay (n=4). Data were normalised to serum-free medium (con) which was set 1 (**p< 0.01; ***p< 0.001 compared to serum free medium). **C)** Expression of acetylated p53 (K382), p21 protein and cleavage of caspase-3 were analysed in HepG2 cells treated with EX527+TSA for 24h. GAPDH was used as loading control. One representative blot out of 3 independent experiments is shown.

Resveratrol differentially regulates NAMPT enzymatic activity in hepatocarcinoma cells and primary human hepatocytes

Based on our findings that an inhibition of NAMPT and SIRT1 activity induced growth arrest and apoptosis in hepatocarcinoma cells we then asked whether resveratrol would also affect NAMPT enzyme activity as well as intracellular NAD levels. We found that resveratrol differentially regulated NAMPT activity in hepatocarcinoma cells (Fig.5A) and primary hepatocytes (Fig.5B) without affecting NAMPT protein expression (Fig.5C,D). We measured a dose-dependently decreased NAMPT activity in HepG2 cells ([100μM] -38.9±14.0%, $p<0.01$) (Fig.5A) and in Hep3B cells ([100μM] -38.5± 9.4%, $p<0.05$) (Fig.S4). In contrast, NAMPT enzymatic activity in primary hepatocytes significantly increased by +64.7±13.8% ($p< 0.05$) after stimulation with 100 μM resveratrol (Fig.5B).

We then measured the NAD level after resveratrol treatment in HepG2 cells and found a trend towards reduction ([con] 2.0±0.4 μmol NAD/ g total protein, [100μM] 1.5±0.2 μmol NAD/g total protein) (Fig.5E). In contrast, intracellular NAD levels in primary hepatocytes were increased by resveratrol ([con] 1.7±0.3 μmol NAD/g total protein, [50μM] 6.4±2.5 μmol NAD/g total protein ($p< 0.05$), [100μM] 5.4±1.7 μmol NAD/g total protein) (Fig.5F).

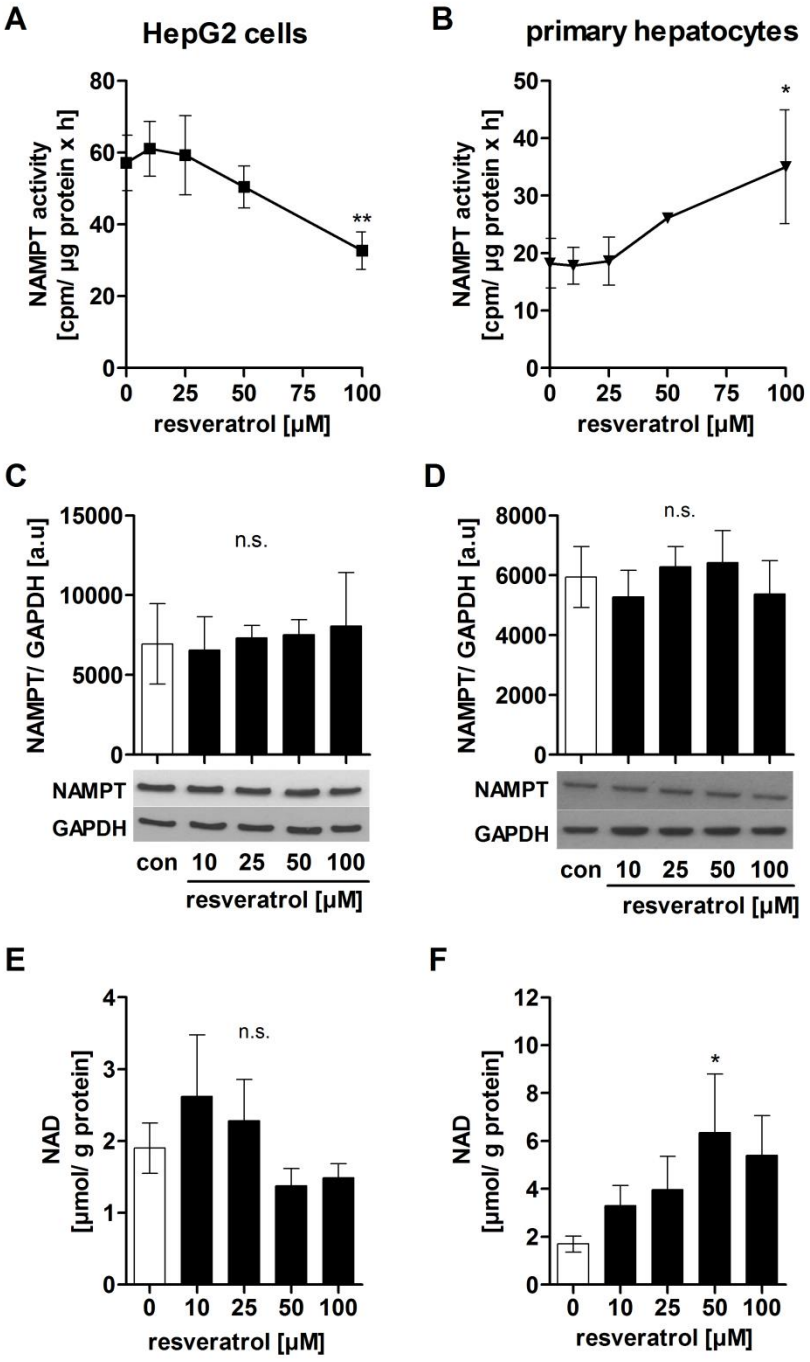

Figure 5. Resveratrol differentially regulates NAMPT and NAD levels in HepG2 cells and primary human hepatocytes. Cells were stimulated with resveratrol or serum-free medium (con) for 24 h. For measuring NAMPT enzymatic activity in **A)** HepG2 cells and (n=4) **B)** primary human hepatocytes (n=3), 50µg of protein lysate was used for the assay and incubated for 1h. Counts (cpm) were normalised to µg total protein. Lysates from **C)** HepG2 cells (n=3) and **D)** primary human hepatocytes (n=3) were used to measure NAMPT protein levels by Western Blot. Determination of intracellular NAD levels in **E)** HepG2 cells (n=6) and **F)** primary human hepatocytes (n=4). NAD levels were normalised to total protein amount in each sample. Data are shown as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p< 0.05; **p< 0.01).

Resveratrol differentially regulates p53 acetylation and SIRT1 protein in hepatocarcinoma cells and primary human hepatocytes

We further addressed whether resveratrol could influence p53 acetylation at lysine residue 382, a main target site of SIRT1[16,17,34], and demonstrated that resveratrol treatment of HepG2 cells increased acetylation of p53 ([50μM] 12.8-fold, [100μM] 13.4-fold) (Fig.6A). As positive control for SIRT1 inhibition, we used the specific SIRT1 inhibitor EX527 [34,37] (Fig.6A). In contrast, primary human hepatocytes from different donors showed a trend towards reduced p53 acetylation after resveratrol stimulation (Fig.6B). Since the acetylation of p53 activates its transcriptional activity, we analysed the expression of the p53 downstream target p21/WAF1/Cip1, which functions as a regulator of cell cycle progression. In correspondence to the acetylation state of p53 we found increased expression of p21 mRNA (Fig.S5A) and protein in HepG2 cells (Fig.6A).

Primary human hepatocytes from different donors showed variable results with either no changes in p21 protein expression or a p21 down regulation (Fig.6B, Fig.S5B). Nonetheless, we can exclude an activation of p53 in primary human hepatocytes. Furthermore, SIRT1 protein levels in HepG2 cells were reduced at 100μM resveratrol ($p<0.01$) (Fig.6C) whereas primary human hepatocytes showed a trend towards increased SIRT1 protein expression at the same dose of resveratrol (Fig.6D). Due to the variability of primary hepatocytes the changes were not significant.

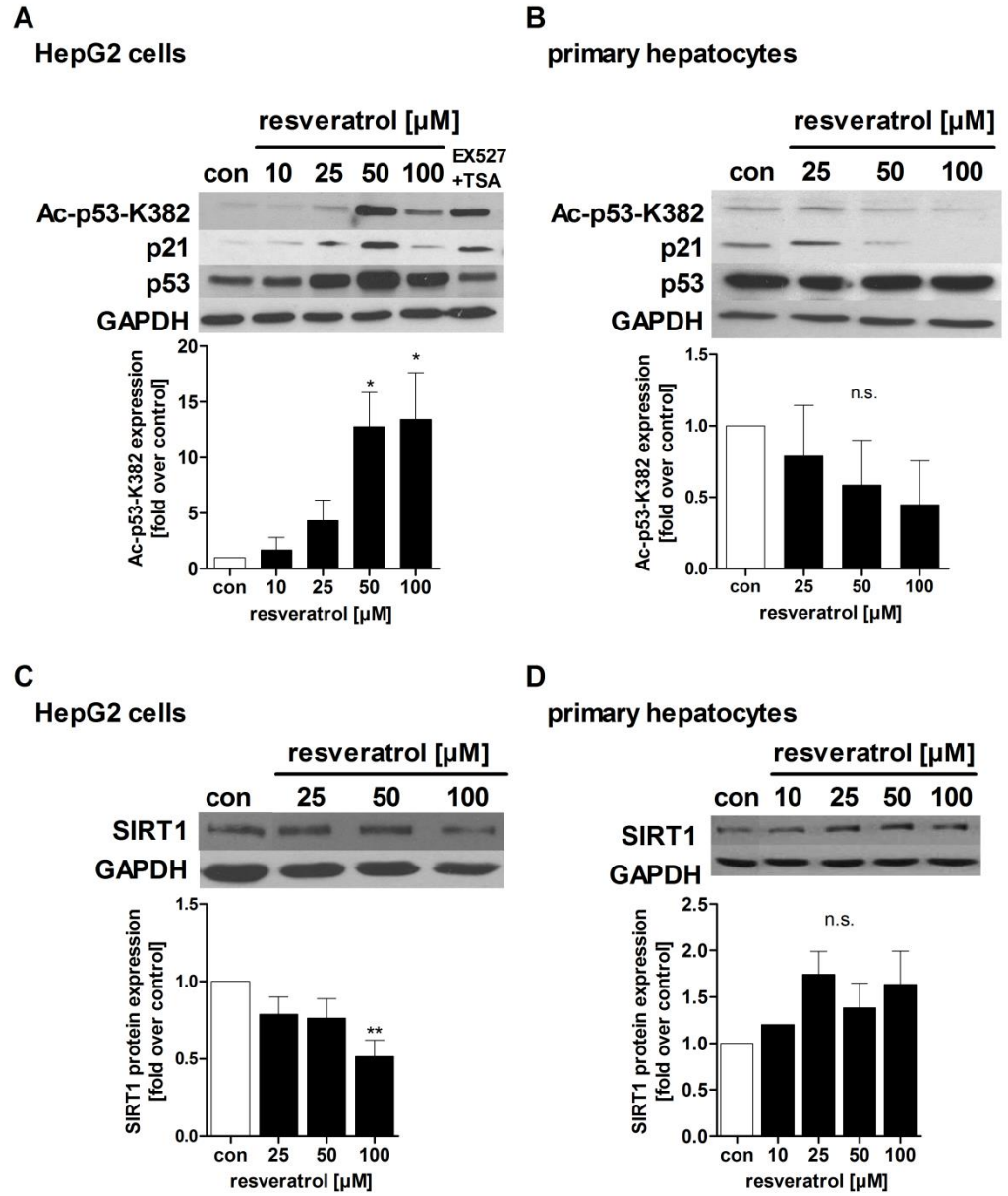

Figure 6. Resveratrol differentially regulates p53 acetylation and SIRT1 protein level in HepG2 cells and primary human hepatocytes. Acetylation of p53 (K382) in **A)** HepG2 cells (n=4) and **B)** primary human hepatocytes (n=3) was evaluated by Western Blot. Densitometric analysis of at least three independent experiments is shown. As a downstream target of acetylated and activated p53, the expression of p21 was analysed by Western Blot. As positive control for SIRT1 inhibition, EX527+TSA was used. SIRT1 protein expression was analysed by Western Blot in **C)** HepG2 cells and **D)** primary hepatocytes and densitometric analysis was performed. GAPDH was used as loading control, respectively. One representative blot out of at least 3 independent experiments is shown. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*p< 0.05, **p< 0.01, n.s. not significant).

Resveratrol induces NAMPT release in HepG2 cells

Since NAMPT was found to be released from hepatocytes [43] we determined NAMPT concentrations in supernatants from resveratrol-treated HepG2 cells and primary hepatocytes. We measured significantly increased amounts of extracellular NAMPT in the supernatant of HepG2 cells treated with 50μM (4-fold) and 100 μM (19.8-fold) resveratrol (Fig.7A) ([con] 0.4±0.2 ng NAMPT/mg total protein, [50μM] 1.6±0.7 ng NAMPT/mg total protein, [100μM] 7.9±1.3 ng NAMPT/mg total protein, $p< 0.001$). We postulated that *NAMPT* mRNA expression may be increased following resveratrol exposure in HepG2 cells to maintain a steady-state of intracellular NAMPT protein level. We found a significantly increased *NAMPT* gene expression after stimulation with 50 μM (1.8-fold, $p< 0.05$) and 100 μM (1.7-fold, $p<0.05$) resveratrol in HepG2 cells (Fig.7C).

NAMPT release and *NAMPT* mRNA expression in primary human hepatocytes were not influenced by resveratrol (Fig.7B,D). In parallel, a cytotoxicity assay was performed to verify that the increase of extracellular NAMPT levels was not due to leakage from damaged cells (Fig.S3B). We then asked at which time point NAMPT release from HepG2 cells started. We found that there was a time- and dose- dependent release of NAMPT already starting after 6h of resveratrol exposure (Fig.S3C). NAMPT is known as a protein with dual function- an enzyme and a cytokine-like function. We asked whether NAMPT that is released after resveratrol exposure could lack NMN biosynthetic action. We found a remarkable decrease in extracellular NAMPT activity by 72.3±11.9% ($p< 0.001$) compared to control cells in serum-free medium (Fig.7E).

NMN does not protect against resveratrol-induced apoptosis in hepatocarcinoma cells

Next, we investigated whether NMN would be able to ameliorate resveratrol-mediated effects in HepG2 cells. Interestingly, NMN did not protect from resveratrol-induced cell cycle arrest and apoptosis in hepatocarcinoma cells (Fig.S6A,B,C,D). Further, NMN was not able to abrogate p53 hyperacetylation after resveratrol treatment and to decrease resveratrol-induced NAMPT release in HepG2 cells (Fig.S6E,F).

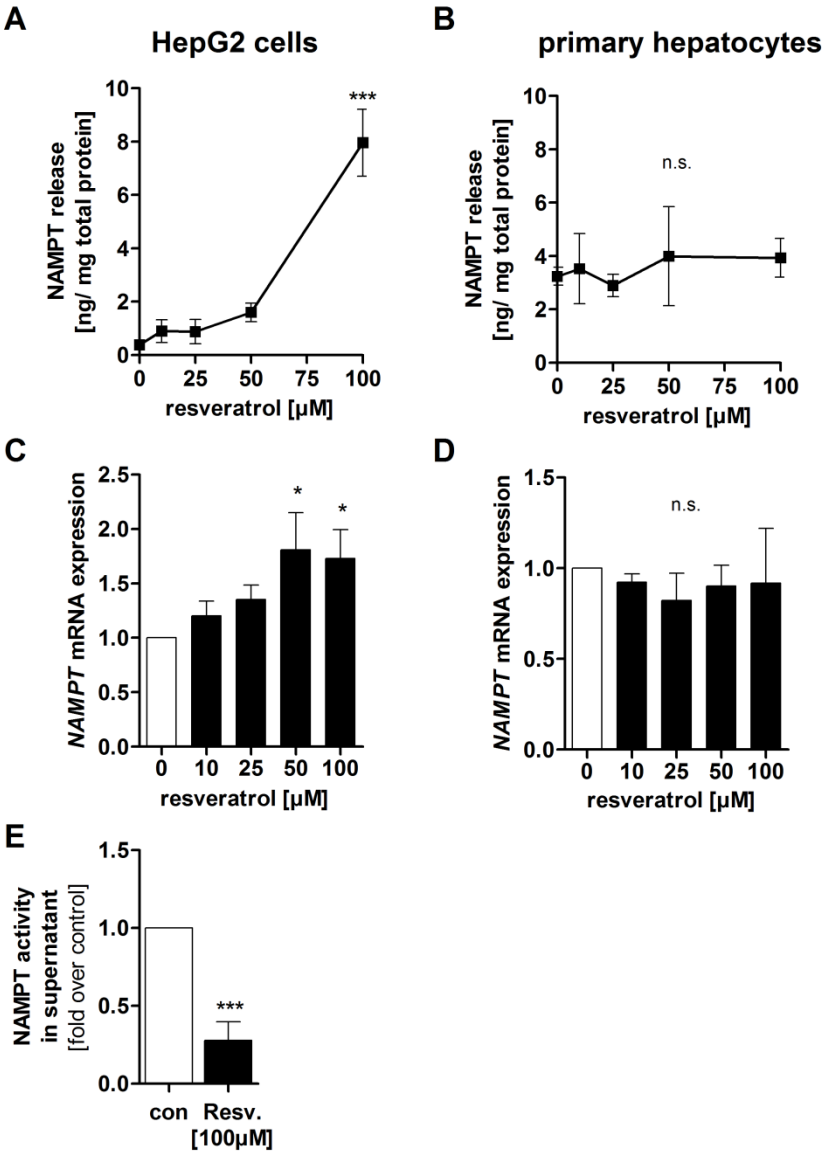

Figure 7. Effects of resveratrol on NAMPT release and NAMPT mRNA expression. Cells were stimulated with resveratrol in serum-free medium for 24 h. Supernatants of resveratrol treated **A)** HepG2 cells (n=7) and **B)** primary human hepatocytes (n=3) were used for quantifying extracellular NAMPT protein amount using a specific eNAMPT ELISA. eNAMPT protein concentration was normalised to the total protein amount. *NAMPT* mRNA expression in resveratrol treated **C)** HepG2 cells (n=5) and **D)** primary human hepatocytes (n=4) was quantified by qRT-PCR and normalised to housekeeping genes. *NAMPT* gene expression was then related to its expression in serum-free control medium (0), which was set 1. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*$p < 0.05$; ***$p < 0.001$; n.s. not significant). **E)** Supernatant of resveratrol [100µM] or serum-free medium (con) treated HepG2 cells was used to measure NAMPT enzymatic activity and extracellular NAMPT protein levels. Counts (cpm) measured by NAMPT enzyme assay were referred to densitometric data of NAMPT protein levels in the supernatant of the same sample. Data were then normalised to serum-free control medium which was set 1. Data are shown as mean± SEM. The difference between these two groups was evaluated using unpaired Student's *t*-test (***$p < 0.001$).

SIRT1 inhibition decreases NAMPT activity and induces NAMPT release

Given that resveratrol increased p53 acetylation (K382), downregulated NAMPT activity and induced NAMPT secretion, we asked whether an inhibition of SIRT1 by EX527 would exert the same effects. Indeed, our data revealed that HepG2 cells treated with EX527 showed the same cellular responses as cells stimulated with resveratrol, such as decreased NAMPT enzymatic activity (-40.3±11.5%, $p<0.05$) (Fig.8A) and slightly reduced intracellular NAD level (Fig.8B). Further, as observed in resveratrol-treated HepG2 cells, extracellular NAMPT levels were increased upon SIRT1 inhibition (Fig.8C).

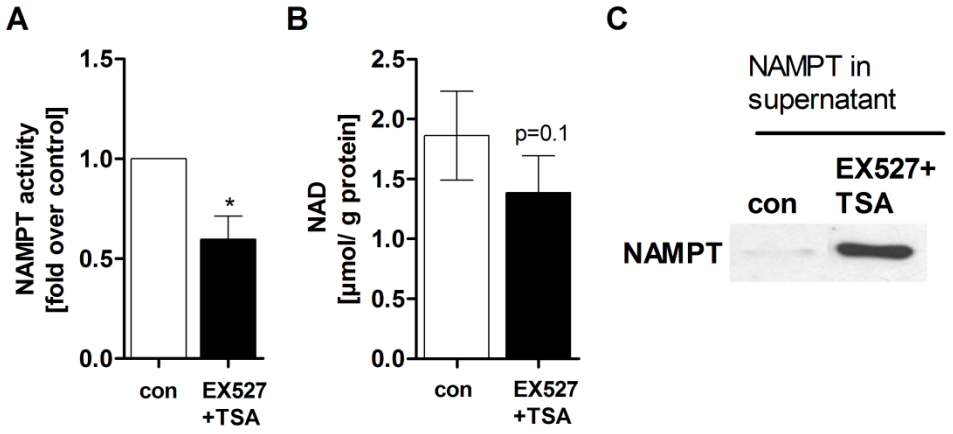

Figure 8. SIRT1 inhibition downregulates NAMPT activity and induces NAMPT release in HepG2 cells. HepG2 cells were treated with EX527+TSA [20µM EX527+ 1µM TSA] or serum-free medium (con) for 24h. Measurement of **A)** NAMPT enzymatic activity (n=3). Counts (cpm) were normalised to µg total protein in each sample (*$p<0.05$). **B)** NAD level were determined by HPLC (n=5) and normalised to total protein amount in each sample. **C)** Supernatant of EX527 treated HepG2 cells was used for determination of eNAMPT level. One representative Western blot out of 3 independent experiments is shown.

SIRT1 overexpression abrogated resveratrol- induced p53 hyperacetylation, NAMPT release and S-phase arrest

Since resveratrol and NMN co-treatment did not augment resveratrol-induced p53 hyperacetylation in hepatocarcinoma cells, we tried to overcome this effect by transiently overexpressing SIRT1 in HepG2 cells (Fig.9A). Our data revealed that SIRT1 overexpression significantly decreased resveratrol- induced p53 hyperacetylation ([50µM] -76.6±6.5%, [100µM] -69.9±15.9%, $p<0.05$) and its transcriptional activity in HepG2 cells (Fig.9B). We then investigated whether a SIRT1 overexpression would be able to abrogate resveratrol-induced NAMPT secretion in HepG2 cells. SIRT1 overexpressing HepG2 cells treated with 100µM resveratrol led to decreased eNAMPT levels in the supernatant compared to mock-transfected cells treated with resveratrol alone (Fig.9C). This suggests

that SIRT1 may play a crucial role in the mechanism of resveratrol-induced NAMPT secretion. Reduction in cell viability upon resveratrol treatment [100µM] was not abolished by SIRT1 overexpression (Fig.9D) indicating that apoptosis inducing factors were still activated and not dependent on SIRT1. However, the resveratrol- induced cell cycle arrest in the S-phase was significantly decreased after SIRT1 overexpression ([con] 7.4±0.9%, [con+25µM] 28.1±2.6%, [Flag-SIRT1+25µM] 18.5±3.8%; [con+50µM] 26.6±3%, [Flag-SIRT1+50µM] 17.1±3.2%) (Fig.9E).

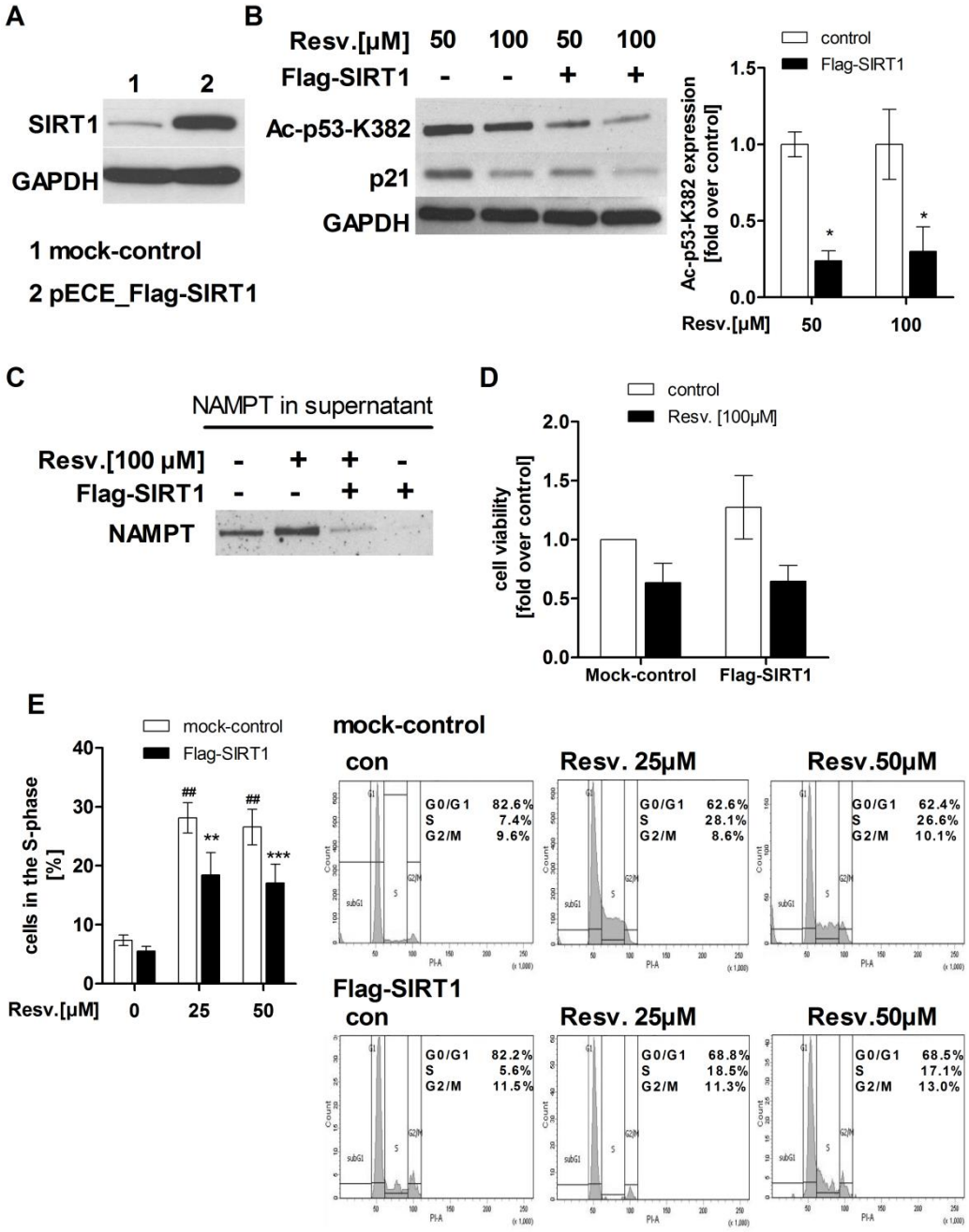

Figure 9. SIRT1 overexpression in HepG2 cells reversed resveratrol-induced SIRT1 inhibition, NAMPT release and S-phase arrest. A) SIRT1 was transiently overexpressed in HepG2 cells [2.0µg plasmid/ 0.5x10^6 cells] using the expression vector pECE_Flag-SIRT1 from

addgene (plasmid 1791; [35]). Lysates of cells transfected with the empty vector pECE (mock-control) (1) or pECE Flag-SIRT1 vector (2) were used for Western Blot analysis. **B)** mock-transfected (mock-control) and Flag-SIRT1 transfected HepG2 cells were stimulated with resveratrol [50, 100μM Resv.] for 24h and Western Blot analysis of acetylated p53 (K382), p21 and GAPDH was performed. Densitometric anaylsis of acetylated p53 of three independent Western Blots is shown. Data were normalised to non-transfected HepG2 cells stimulated with resveratrol alone which was set 1. **C)** To analyse the effect of SIRT1 overexpression on resveratrol-induced NAMPT release, supernatant of mock-transfected and Flag-SIRT1 transfected HepG2 cells stimulated with or without resveratrol [100μM] were used to measure eNAMPT level. One representative Western blot out of 3 independent experiments is shown. **D)** Cell viability of mock-transfected and Flag-SIRT1 transfected HepG2 cells treated with resveratrol [100μM] (black bars) was measured using WST-1 assay (n=3). Data were normalised to untreated mock-control which was set 1. **E)** mock-transfected (white bars) and Flag-SIRT1 transfected HepG2 cells (black bars) were stimulated with resveratrol [25, 50μM] for 24h. Percentage of cells in the S-phase were measured by PI staining and FACS analysis. All data are shown as mean± SEM (n=4). The difference between two groups was evaluated using unpaired Student's *t*-test (##p<0.01 mock-transfected cells compared to mock-transfected cells treated with resveratrol (white bars, mock-control), **p<0.01, ***p< 0.001 Flag-SIRT1 transfected cells treated with resveratrol (black bars) compared to resveratrol-treated mock-transfected cells (white bars).

4.4 Discussion

There is growing *in vitro* and *in vivo* evidence demonstrating the inhibitory effects of resveratrol on liver cancer [44–46]. It is known that resveratrol affects numerous signal transduction pathways associated with tumorigenesis [47]. However, the mechanisms how resveratrol selectively modulates proliferation and apoptosis in tumor cells are not fully understood. A recent study demonstrated that resveratrol has the chemical structure to inhibit the activity of different human histone deacetylases (HDACs), important transcriptional and post-translational regulators [48]. We investigated the molecular mechanisms of resveratrol-induced reduction of cell viability in human hepatocellular carcinoma cells and compared the results with non-cancerous primary human hepatocytes. We found that resveratrol selectively induced apoptosis in HepG2 and Hep3B cells, but not in primary hepatocytes. Our data confirm the apoptotic effects of resveratrol on hepatocarcinoma cells independent of p53 function. Furthermore, several other studies reported that resveratrol induced p53-independent apoptosis in tumor cells [49,50], indicating that p53 is not an absolute requirement for the apoptotic effect of resveratrol. In contrast, we found an arrest of cells in the S- and G2/M-phase of the cell cycle only in p53 wild-type HepG2 cells and not in Hep3B cells lacking p53, which was also shown by other groups [7,51,52].

Our study revealed that NAMPT and SIRT1 were expressed in an opposite way in hepatocarcinoma cells and primary hepatocytes and were differentially regulated by resveratrol. Other groups found SIRT1 expression to be significantly elevated in hepatocellular carcinoma (HCC) compared to non-cancerous tissues, the expression levels

correlated with tumor grades and predicted poor prognosis. SIRT1 was shown to promote tumorigenesis in HCC, and inhibition of SIRT1 consistently suppressed the proliferation of HCC cells *in vitro* or *in vivo* via the induction of cellular senescence or apoptosis [53–56]. The expression and role of NAMPT in HCC has not been characterised so far. In contrast to findings in other cancer cell types [18], we found that hepatocarcinoma cells express lower levels of NAMPT compared to non-cancerous primary hepatocytes. However, we found that HepG2 cells possess a higher basal NAMPT activity than primary hepatocytes, which goes in line with the comparable NAD levels in both cell types despite weaker NAMPT expression in hepatocarcinoma cells.

Under basal conditions primary hepatocytes released higher amounts of NAMPT into the supernatant than HepG2 cells. As shown by our group, HepG2 cells and primary human hepatocytes constitutively release NAMPT in its dimeric, enzymatically active form [43]. Presumably, due to the higher amount of cellular NAMPT protein in primary human hepatocytes compared to hepatocarcinoma cells, NAMPT is constitutively more released from primary hepatocytes leading to higher basal eNAMPT level. However, we cannot completely preclude, that due to necrosis of fragile or dead primary hepatocytes, proteins are released into the supernatant.

There are several reports showing that resveratrol acts as a natural SIRT1 activator [10,57–60]. We observed increased NAMPT activity and intracellular NAD levels in primary hepatocytes providing evidence for resveratrol as SIRT1 activator in non-cancerous cells. However, recent data showed that resveratrol is not a direct activator of SIRT1 and therefore some mediators are may be involved in this interplay [48,61–63]. Moreover, little is known about NAMPT and SIRT1 regulation by resveratrol in cancer cells. A recent report showed that SIRT1 inhibition is involved in resveratrol-induced cell death in Hodgkin lymphoma (HL)-derived L-428 cells [11]. Additionally, neuroblastoma cells treated with resveratrol also underwent apoptosis and showed a downregulaton of SIRT1 [64]. In our study, resveratrol-stimulated HepG2 cells showed similar responses like cells treated with the SIRT1 inhibitor EX527. Thus, we assume that resveratrol is rather acting as a NAMPT and SIRT1 inhibitor in hepatocarcinoma cells. However, there are several reports demonstrating other mechanisms leading to p53 acetylation by resveratrol [65,66].

Our findings indicate that NMN did not ameliorate resveratrol-induced effects on apoptosis, cell cycle arrest and NAMPT release, suggesting that the availability of NAD is not a limiting factor in this scenario. A variety of posttranslational modifications in SIRT1

N- and C-terminal extensions have been reported, effecting SIRT1 enzyme activity and protein interactions [67,68]. Our findings raise the possibility that, in some cases, the regulation of SIRT1 by other proteins may be more important than NAD availability. Increase in p53 transcriptional activity and induction of S-phase arrest observed upon treatment with resveratrol were abrogated upon SIRT1 overexpression. However, SIRT1 overexpression was not able to augment reduced cell viability in HepG2 cells under high resveratrol concentrations. Resveratrol affects a multitude of other signal transduction pathways associated with apoptotic mechanisms and transcriptional regulation [66,69] that are still activated and not SIRT1 dependent [64]. Thus, these collective activities, rather than just a single effect, may account for the anticancer properties of resveratrol. However, our data give evidence that resveratrol regulates NAMPT activity in cancer cells and non-cancerous cells. Resveratrol could regulate NAMPT enzymatic activity by at least two hypothetical mechanisms: i) by direct interaction inducing conformational changes that lead to alterations of enzymatic activity, or ii) by inducing a posttranslational modification of NAMPT. Our study revealed that SIRT1 inhibition downregulates NAMPT activity and induces NAMPT secretion. This provides the basis for further mechanistic studies on NAMPT-SIRT1 interaction and their regulation.

Further, we found a time- and dose-dependent NAMPT release after resveratrol stimulation of HepG2 cells which was associated with increased *NAMPT* mRNA expression. The association of increased NAMPT release and mRNA expression has also been shown by Kover *et al.* in human islets [70]. eNAMPT has been described to act as a cytokine (as pre-B cell colony enhancing factor, PBEF)[71] or as an adipokine (visfatin)[72,73] but also has extracellular enzymatic function to yield NMN [74]. To our knowledge, for the first time our data point to SIRT1 as regulator of NAMPT secretion and NAMPT enzymatic activity in the supernatant. We could show that the resveratrol-induced NAMPT release was significantly reduced after SIRT1 overexpression, indicating a crucial role for SIRT1 in resveratrol-mediated NAMPT secretion.

In summary, our study revealed that resveratrol selectively induced p53-independent cell death in hepatocarcinoma cells and differentially regulated NAMPT and SIRT1 in cancer cells and non-cancerous cells. Our data give evidence that in contrast to normal hepatocytes, resveratrol does not act as a NAMPT and SIRT1 activator in hepatocarcinoma cells. However, it remains to be investigated whether NAMPT interacts with SIRT1 and how it is regulated by resveratrol or other mediators and linked to cellular

metabolism and apoptosis. This will provide novel insights concerning the potential of NAMPT and SIRT1 as therapeutic targets in hepatocellular carcinoma.

4.5 Acknowledgments

Primary human hepatocytes were kindly provided by the "virtual liver" program, a major national initiative on Systems Biology of the Liver. FK866 was kindly provided by TopoTarget A/S, Copenhagen, Denmark. We thank Prof. Dr. Kurt Engeland for providing Hep3B cells and our technicians Anja Barnikol-Oettler and Sandy Richter as well as Doris Mahn and Frank Struck for excellent work.

Detailed author contributions can be found at the end of this work.

4.6 Supplementary Material

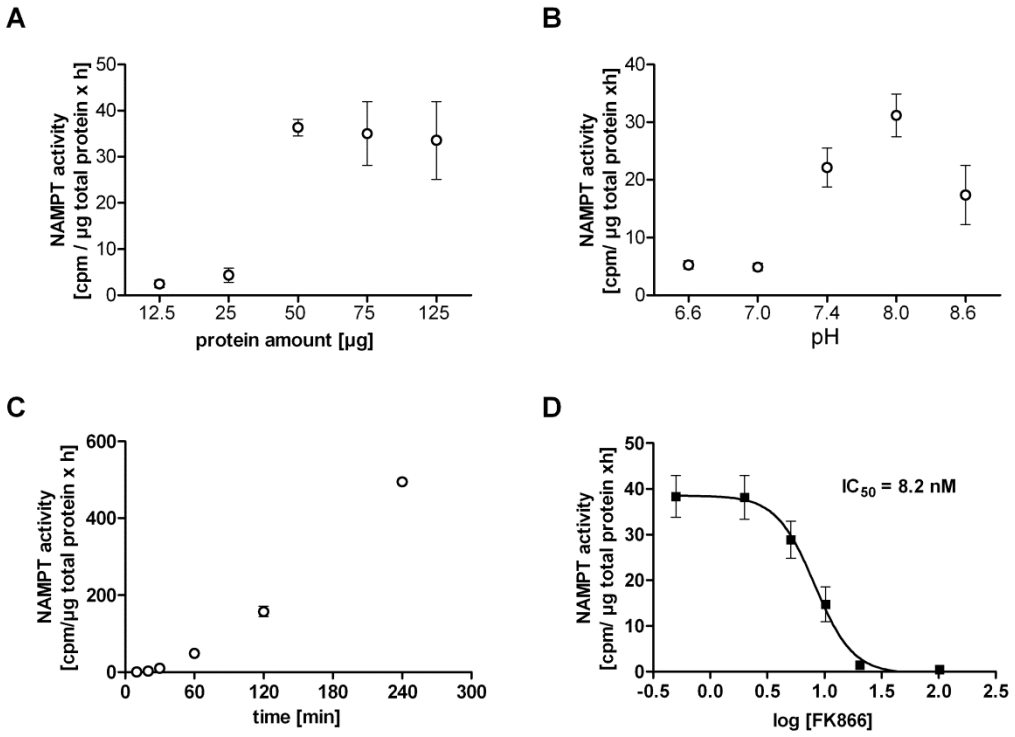

Figure S1. Establishment of parameters for a NAMPT enzymatic assay. Assay conditions, such as **A)** protein amount, **B)** pH value and **C)** incubation time were optimized for measuring NAMPT enzymatic activity. **D)** We validated the assay performance by adding the specific NAMPT inhibitor FK866 to the lysate before measuring NAMPT activity. As expected, FK866 induced a dose-dependent decrease in NAMPT activity with an IC_{50} value of 8.2 nM**.** Experiments were performed in HepG2 cells. Data are presented as mean± SEM.

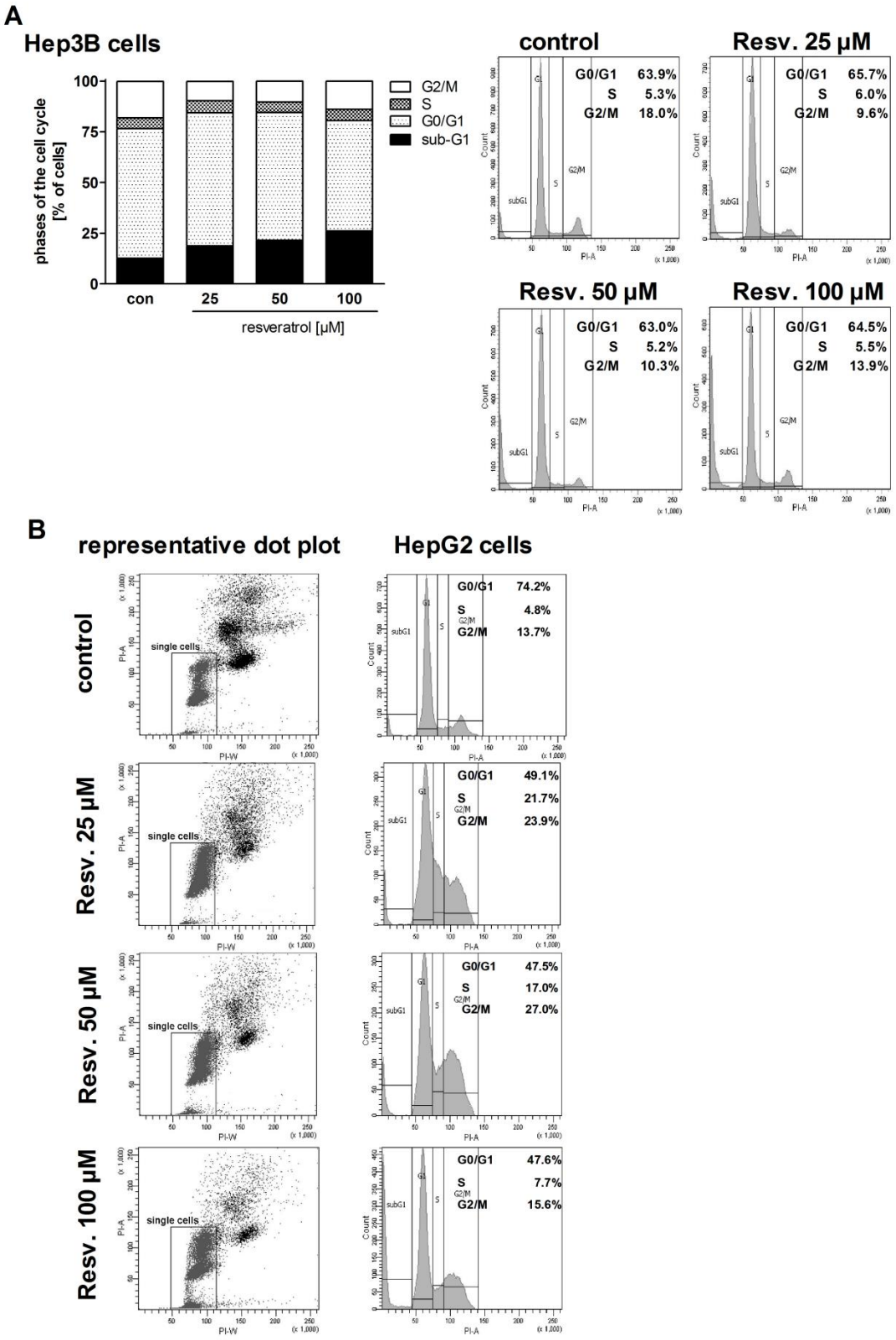

Figure S2. Cell cycle arrest in Hep3B and a representative dot plot of HepG2 cells. A) PI staining of cell cycle distribution of Hep3B cells (n=2) stimulated with different concentration of resveratrol [25/50/100µM] for 24h**. B)** A representative dot plot of cell cycle analysis of HepG2 cells. The left plot shows pulse width versus area; this is the plot used to distinguish between single cells and aggregates. Single cells have been gated and a FL2-Area histogram has been drawn and formatted to show only the events inside of the single cell region.

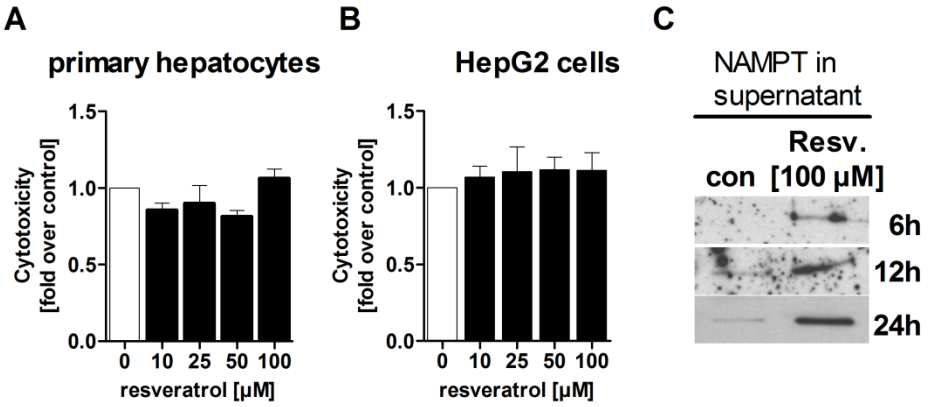

Figure S3. Resveratrol does not have cytotoxic effects on HepG2 cells and primary human hepatocytes. HepG2 cells and primary human hepatocytes were stimulated with resveratrol [10/25/50/100µM] in serum-free medium for 24h and supernatant was used for the ToxiLight Non-destructive Cytotoxicity BioAssay. **A)** Primary human hepatocytes (n=3) and **B)** HepG2 cells (n=3) showed no cytotoxic effects after stimulation with resveratrol. Data are shown as mean± SEM. Statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (n.s. not significant). **C)** Supernatants of resveratrol [100µM] or serum-free medium (con) treated HepG2 cells after 6, 12 and 24h were used to measure extracellular NAMPT levels by Western Blot.

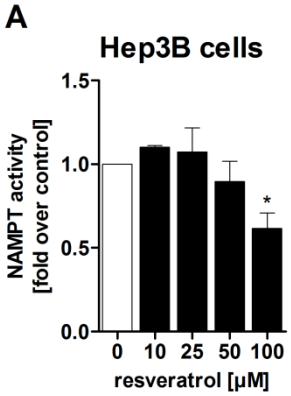

Figure S4. Resveratrol downregulates NAMPT enzymatic activity in Hep3B cells. Hep3B cells were stimulated with resveratrol [10/25/50/100µM] in serum-free medium for 24h. NAMPT enzymatic activity was measured by the conversion of ^{14}C- labelled nicotinamide to ^{14}C-NMN (see Material and Methods). Counts (cpm) were normalised to µg total protein in each sample measured by BCA protein assay. Data are represented as mean± SEM and statistical analysis was performed using one-way ANOVA and the Bonferroni post hoc test (*$p < 0.05$).

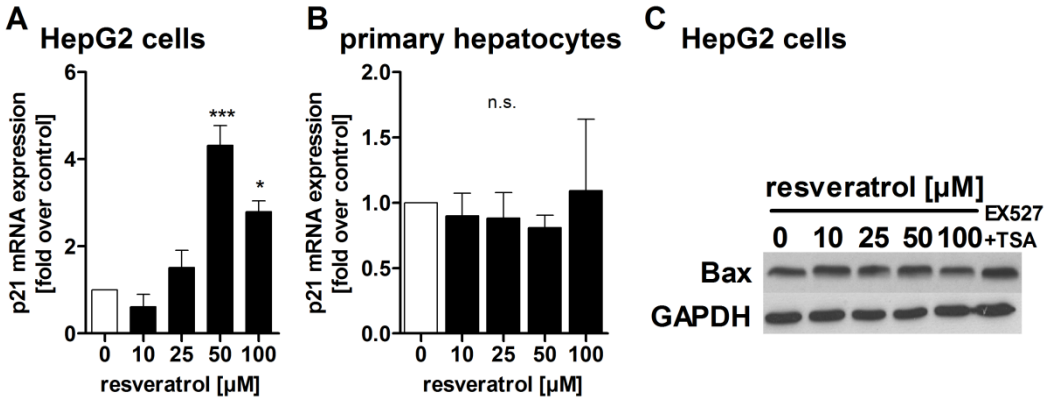

Figure S5. p21 and Bax expression in HepG2 cells and primary human hepatocytes. HepG2 cells and primary human hepatocytes were stimulated with resveratrol [10/25/50/100µM] in serum-free medium (0) for 24h. *p21* mRNA expression in **A)** HepG2 cells (n=3) and **B)** primary human hepatocytes (n=4). **C)** Lysates of HepG2 cells (n=3) were used for Western Blot analysis of Bax protein expression. GAPDH was used as loading control. One representative blot out of 3 independent experiments is shown.

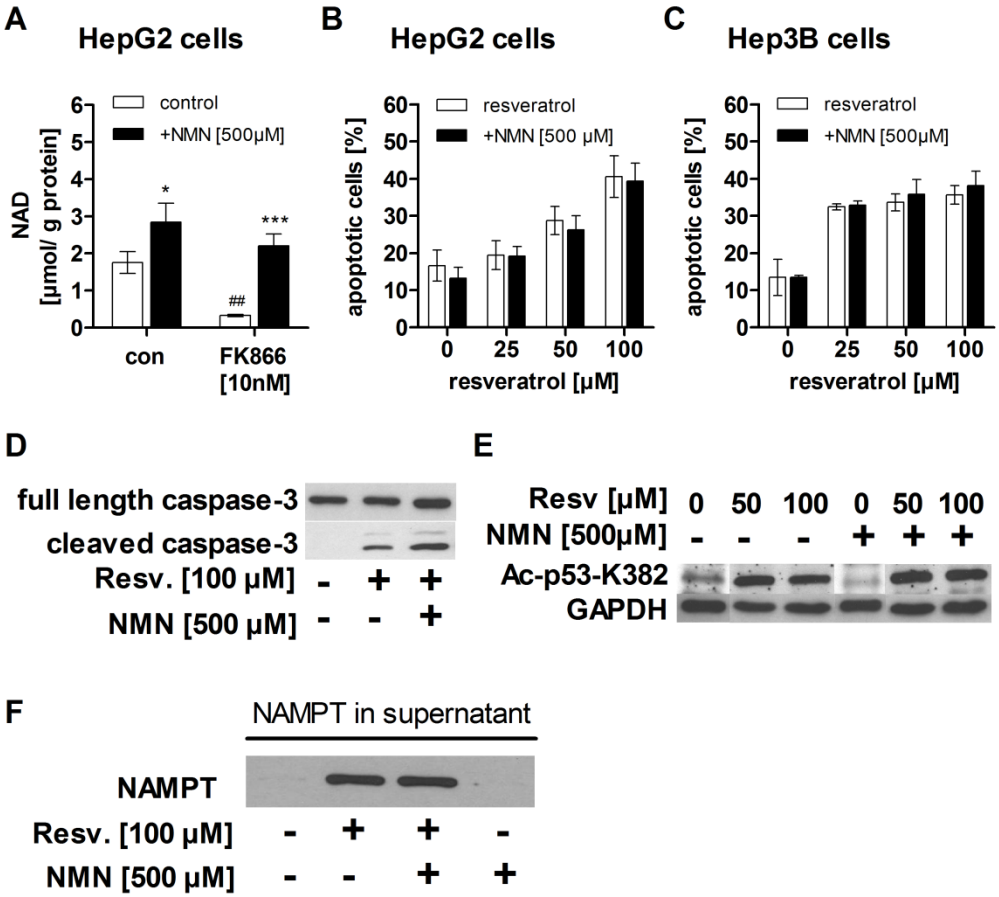

Figure S6. NMN does not ameliorate resveratrol-mediated effects on cell viability, NAMPT activity, NAMPT release and p53 hyperacetylation. Given that resvertarol down-regulates NAMPT and increases p53 acetylation in hepatocarcinoma cells which was absent in primary hepatocytes we hypothesised that the administration of NMN, the reaction product of NAMPT and a precursor of NAD, is able to ameliorate the resveratrol-mediated effects by increasing SIRT1 activity. At the beginning, we tested whether HepG2 cells are able to utilize exogenous NMN [500µM] and to synthesize NAD. Therefore, we stimulated the cells with FK866 [10nM] to inhibit NAMPT activity and co-stimulated the cells with NMN [500µM]. We could show that FK866 depleted the intracellular NAD levels by -79.4±3.3% in HepG2 cells which could be restored by NMN supplementation. **A)** NAD levels of HepG2 cells (n=5) treated with the NAMPT inhibitor FK866 [10nM] (white bars) in serum-free medium (con) and NMN [500µM] (black bars) for 24h. Annexin V/PI apoptosis assay of **B)** HepG2 cells (n=3) and **C)** Hep3B cells (n=2) treated with resveratrol [25/50/100µM] in serum-free medium and co-stimulated with NMN [500µM] for 24h. An+ and An+/PI+ cells were considered apoptotic. Data are represented as mean± SEM. Differences between two groups were evaluated using unpaired Student's *t*-test (resvertarol (white bar) compared to resveratrol +NMN (black bar)). Then **D)** Western Blot analysis of cleaved caspase-3, **E)** acetylated p53 (K382) and **F)** eNAMPT levels in supernatant of these cells were performed. One representative blot out of at least 3 independent experiments is shown.

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PUBLICATIONS AND PRESENTATIONS

Publications

first-author publications:

S. Schuster*, M. Penke*, T. Gorski, R. Gebhardt, T.S. Weiss, W. Kiess, A. Garten. FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signalling in hepatocarcinoma cells. Biochem Biophys Res Commun. 2015 Mar 6;458(2):334-40.
* These authores contributed equally to this work.

S. Schuster, M. Penke, T. Gorski, S. Petzold-Quinque, G. Damm, R. Gebhardt, W. Kiess, A. Garten. Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes. PLoS One. 2014 Mar 6;9(3):e91045.

S. Schuster*, C. Hechler*, C. Gebauer, W. Kiess, J. Kratzsch. Leptin in maternal serum and breast milk: association with infants' body weight gain in a longitudinal study over 6 months of lactation. Pediatr Res. 2011 Dec;70(6):633-7.
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Co-author publications:

A. Garten, **S. Schuster**, M. Penke, T. Gorski, T. deGiorgis, W. Kiess, Physiological and pathophysiological roles of NAMPT and NAD metabolism. Nature Reviews Endocrinology, *under review* (Review)

M. Penke, PS Larsen, **S. Schuster**, T. Gorski, A. Meusel, S. Richter, S.G. Vienberg, J.T. Treebak, W. Kiess, A. Garten. Hepatic NAD salvage pathway is up regulated in mice by an excessive supply of lipids. Molecular and Cellular Endocrinology, *in revision*

G. L. Schmid, F. Kässner, H. H. Uhlig, A. Körner, J. Kratzsch, N. Händel, F.P. Zepp, F. Kowalzik, A. Laner, S. Starke, F. K. Wilhelm, **S. Schuster**, A. Viehweger, W. Hirsch, W. Kiess, A. Garten. Sirolimus treatment of severe PTEN hamartoma tumor syndrome: case report and in vitro studies. Pediatr Res. 2014 Apr;75(4):527-34.

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A. Garten, **S. Schuster**, W. Kiess. The insulin-like growth factors in adipogenesis and obesity. Endocrinol Metab Clin North Am. 2012 Jun;41(2):283-95. (Review)

A. Garten, S. Petzold, **S. Schuster**, A. Körner, J. Kratzsch, W. Kiess. Nampt and its potential role in inflammation and type 2 diabetes. Handb Exp Pharmacol. 2011;(203):147-64. (Review)

Poster Presentations

S. Schuster, M. Penke, T. Gorski, S. Richter, A. Garten , W. Kiess, "FK866-Induced NAD Depletion Leads to AMPK Activation and Inhibition of mTOR Signalling", The ENDO 97th Annual Meeting & Expo, San Diego 2015

S. Schuster, M. Penke, T. Gorski, S. Richter, T. deGiorgis, W. Kiess, A. Garten, "FK866-Induced NAD Depletion Leads to AMPK Activation and Inhibition of mTOR Signalling in human hepatoma cells", 13th Leipziger Research Festival, Leipzig 2014

S. Schuster, M. Penke, T. Gorski, A. Barnikol- Oettler, R. Gebhardt, W. Kiess, A. Garten, "Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes", Metabolism, Diet and Disease: Cancer and Metabolism, Washington 2014

S. Schuster, S. Petzold- Quinque, T. Gorski, A. Garten, M. Penke, A. Barnikol-Oettler, S. Laue, R. Gebhardt, W. Kiess, "Resveratrol induces cell death in human cancer cells and targets NAMPT", ESPE (European Society for Pediatric Endocrinology), Leipzig 2012

S. Schuster, S. Petzold- Quinque, A. Garten, M. Penke, A. Barnikol- Oettler, S. Laue, R. Gebhardt, W. Kiess, "Resveratrol negatively influences NAMPT in a hepatocarcinoma cell line without modifying intracellular NAD levels - Does nicotinamide play a more important role?", Keystone: Sirtuins in Metabolism, Aging and Disease, Tahoe City 2012

S. Schuster, S. Petzold-Quinque, A. Garten, M. Penke, T. Gorski, A. Barnikol-Oettler, S. Laue, R. Gebhardt, W. Kiess, "Resveratrol modifies the NAD-key enzyme NAMPT and exerts differential effects in healthy and tumoral hepatocytes", 10th Leipziger Research Festival, Leipzig 2011

S. Schuster, S. Petzold-Quinque, A. Garten, A. Barnikol-Oettler, S. Laue, R. Gebhardt, W. Kiess, "The effect of free fatty acids, high glucose levels and resveratrol as nutriceutical on NAMPT activity", ESPE, Glasgow 2011

S. Schuster, S. Petzold-Quinque, A. Garten, A. Barnikol-Oettler, S. Laue, R. Gebhardt, W. Kiess, "The effects of nutrient excess and resveratrol as nutriceutical on NAMPT activity", The ENDO 93rd Annual Meeting & Expo, Boston 2011

Oral Presentations

S. Schuster, M. Penke, T. Gorski, A. Barnikol- Oettler, S. Petzold- Quinque, R. Gebhardt, W. Kiess, A. Garten, "Resveratrol differently regulates Nicotinamide phosphoribosyltransferase in HepG2 cells and primary human hepatocytes – The role of NAMPT in resveratrol mediated chemotherapeutic effects", FASEB Science Research conference: NAD metabolism & signalling Itasca, USA 2013

S. Schuster, A. Garten, S. Petzold- Quinque, M. Penke, T. Gorski, W. Kiess, "Resveratrol modifies the NAD- key enzyme NAMPT and exerts differential effects in primary hepatocytes and the hepatocellular carcinoma cell line HepG2", 49. DGE Kongress (Deutsche Gesellschaft für Ernährung e.V.), München 2012

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SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, Dipl. Ernährungswissenschaftlerin Susanne Schuster, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für die Arbeit erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zwecke einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

Leipzig, den 27.03.2015

Susanne Schuster

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Author contribution statement, Susanne Schuster
The NAMPT-mediated NAD salvage pathway in cancer cell metabolism and its regulation by resveratrol

Author contribution statement

Title: **Physiological and pathophysiological roles of NAMPT and NAD metabolism**

Journal: Nature Reviews Endocrinology, *under review*

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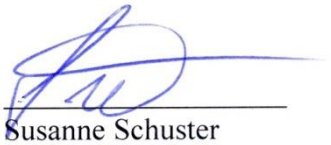

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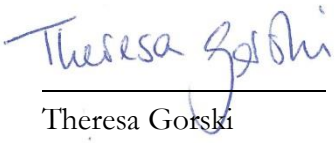

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The NAMPT-mediated NAD salvage pathway in cancer cell metabolism and its regulation by resveratrol

Author contribution statement

Title: **FK866-induced NAMPT inhibition activates AMPK and downregulates mTOR signaling in hepatocarcinoma cells**

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The NAMPT-mediated NAD salvage pathway in cancer cell metabolism and its regulation by resveratrol

Author contribution statement

Title: **Resveratrol differentially regulates NAMPT and SIRT1 in hepatocarcinoma cells and primary human hepatocytes**

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Part Georg Damm:

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- Study funding

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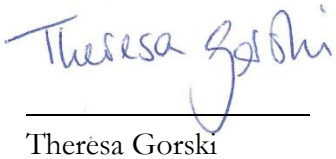
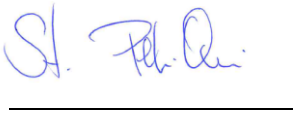


Theresa Gorski



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Author contribution statement, Susanne Schuster
The NAMPT-mediated NAD salvage pathway in cancer cell metabolism and its regulation by resveratrol

Author contribution statement

Title: **Chapter 1: Introduction**

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Bestätigung über die alleinige Urheberschaft von Kapitel 1

Ich, Susanne Schuster, bestätige hiermit die alleinige Urheberschaft des monografisch hinzugefügten Kapitels 1 meiner Dissertation. Dieses Kapitel wurde selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in diesem Kapitel verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht.

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